FORM PTO 1390 (REV. 11-2000) DESIGNATED/ETTER UNDER 25 11 A TIONAL LE US DEPARTMENT OF COMPTE (DO/EO/US) 0459-0571E TRANSMITTAL LE CONCERNING A FILING UNDER 35 U.S.C. 371 PRIORITY DATE CLAIMED INTERNATIONAL APPLICATION NO. INTERNATIONAL FILING DATE -- October 15, 1998 PCT/DK99/00562 October 15, 1999 TITLE OF INVENTION AN IMPROVED METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE IN A * APPLICANT(S) FOR DO/EO/US ARKHAMMAR, Per O.G.; TERRY, Bernard Robert; SCUDDER, Kurt Marshall; BJORN, Sara Petersen ** Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information: This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay 3. examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1). The US has been elected by the expiration of 19 months from the priority date (Article 31). A copy of the International Application as filed (35 U.S.C. 371(c)(2)) is transmitted herewith (required only if not transmitted by the International Bureau). WO 00/23615 has been transmitted by the International Bureau. ı,D is not required, as the application was filed in the United States Receiving Office (RO/US). c. ij An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). is transmitted herewith. a. l has been previously submitted under 35 U.S.C. 154(d)(4) Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)). are transmitted herewith (required only if not transmitted by the International Bureau). have been transmitted by the International Bureau. b. have not been made; however, the time limit for making such amendments has NOT expired. d. have not been made and will not be made. An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). Items 11. to 20. below concern document(s) or information included: An Information Disclosure Statement under 37 CFR 1.97 and 1.98-International Search Report (PCT/ISA/210) 11. An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 12. A FIRST preliminary amendment. 13. X A SECOND or SUBSEQUENT preliminary amendment. A substitute specification. 15. A change of power of attorney and/or address letter. 16. A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825. 17. A second copy of the published international application under 35 U.S.C. 154(d)(4). 18. A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). Other items or information: 20. X 1.) Sixty-eight (68) pages of Sequence Listing 2.) PCT Request (PCT/RO/101) 3.) PCT Substitute Claims Letter w/ International Preliminary Examination Report (PCT/IPEA/409) and claims 4.) Eighteen (18) sheets of Formal Drawings *CELLULAR RESPONSE **THASTRUP, Ole; HAGEL, Grith

U.S. APPLICATION NO (if known, see 37						ATTORNEY'S DOCKET NUMBER		
09/18/	09/807345 PCT/DK99/00562				0459-0571P			
21. The following fees are submitted:						LCULATIONS	PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):								
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO								
and International Search Report not prepared by the EPO or JPO\$1,000.00								
22,000,00								
International preliminary examination fee (37 CFR 1.482) not paid to								
USPTO but International Search Report prepared by the EPO or JPO								
International preliminary evamination for (27 CED 1 402) and and to LIGHTO								
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO								
\$/10.00								
International preliminary examination fee (37 CFR 1.482) paid to USPTO								
but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00								
International preliminary examination fee (37 CFR 1.482) paid to USPTO								
and all claims satisfied provisions of PCT Article 33(1)-(4)								
ENTER APPROPRIATE BASIC FEE AMOUNT =						860.00		
Surcharge of \$130.00 for furnishing the oath or declaration later than 20 30 months from the earliest claimed priority date (37 CFR 1.492(e)).					\$	0		
CLAIMS	NUMBER FILE		NUMBER EXTRA	RATE				
Total Claims	20 - 20 =		0	X \$18.00	\$	0	l	
Independent Claims	1 - 3 =		0	X \$80.00	\$	0		
MULTIPLE DEPENDI	ENT CLAIM(S) (if ar	plicable)	None	+ \$270.00	\$	0		
10.00								
TOTAL OF ABOVE CALCULATIONS = Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are					\$	860.00		
reduced by 1/2.					\$	430.00		
SUBTOTAL =					\$	0		
Processing fee of \$130.00 for furnishing the English translation later than 20 30						^		
months from the earliest claimed priority date (37 CFR 1.492(f)). +					\$	0		
TOTAL NATIONAL FEE =					\$	430.00		
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be						40.00		
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +								
TOTAL FEES ENCLOSED =						470.00		
						Amount to be: refunded	\$	
						charged	\$	
a 🔽 A ahaali in tha an			1					
a. \triangle A check in the amount of \$ $\frac{470.00}{1}$ to cover the above fees is enclosed.								
b. Please charge my Deposit Account. No in the amount of \$ to cover the above fees.								
A duplicate copy of this sheet is enclosed.								
c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any								
overpayment to Deposit Account No. 02-2448.								
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR								
1.137(a) or (b)) must be filed and granted to restore the application to pending status.								
Send all correspondence to:								
Birch, Stewart, Kolasch & Birch, LLP or Customer No. 2292								
P.O. Box 747								
Falls Church, VA 22040-0747								
(703)205-8000						/ / ^	*	
Date: April 12, 2001			F	3y	LN	! well	36.623	
			-	Leonar	d K.	Svensson, #30,3	30	
/cqc						•		

JC02 Rec'd PCT/PTO

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant:

ARKHAMMAR, Per O.G. et al. Conf.:

Int'l. Appl. No.:

PCT/DK99/00562

Appl. No.:

New

Group:

Filed:

April 12, 2001

Examiner:

For:

AN IMPROVED METHOD FOR EXTRACTING

QUANTITATIVE INFORMATION RELATING TO AN

INFLUENCE IN A CELLULAR RESPONSE

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION

Assistant Commissioner for Patents Washington, DC 20231

April 12, 2001

Sir:

The following Preliminary Amendments and Remarks respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Before line 1, insert -- This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/DK99/00562 which has an International filing date of October 15, 1999, which designated the United States of America and was published in English .--

IN THE CLAIMS:

Please amend the claims as follows:

- 3. (Amended) A method according to claim 1, wherein the influence comprises contact between the mechanically intact or permeabilised living cells and a chemical substance and/or incubation of the mechanically intact or permeabilised living cells with a chemical substance.
- 4. (Amended) A method according to claim 1, wherein the cells comprise a group of cells contained within a spatial limitation.
- 5. (Amended) A method according to claim 1, wherein the cells comprises multiple groups of cells contained within multiple spatial limitations.
- 6. (Amended) A method according to claim 1, wherein the spatial limitations are spatial limitations arranged in one or more arrays on a common carrier.
- 8. (Amended) A method according to claim 1, wherein the redistribution results in quenching of fluorescence, the quenching being measure as a decrease in the intensity of the fluorescence.

- 9. (Amended) A method according to claim 1, wherein the redistribution results in energy transfer, the energy transfer being measure as a change in the intensity of the luminescence.
- 10. (Amended) A method according to claim 1, wherein the intensity of the light being recorded is a function of the fluorescence lifetime, polarization, wavelength shift, or other property which is modulated as a result of the underlying cellular response.
- 11. (Amended) A method according to claim 1, wherein the light to be measured passes through a filter which selects the desired component of the light to be measured and rejects other components.
- 12. (Amended) A method according to claim 1, wherein the fluorescence comes from a fluorophore encoded by and expressed from a nucleotide sequence harboured in the cells.
- 13. (Amended) A method according to claim 1, wherein the fluorescence comes from a luminescent polypeptide, such as GFP.
- 14. (Amended) A method according to claim 1, wherein the luminescent polypeptide could be a GFP selected from the group consisting of green fluorescent proteins having the F64L such as F64L-GFP, F64L-Y66H-GFP, F64L-S65T-GFP, and EGFP.

- 15. (Amended) A method according to claim 1, wherein the cells are selected from the group consisting of fungal cells, such as yeast cells; invertebrate cells including insect cells; and vertebrate cells, such as mammalian cells.
- 17. (Amended) A method according to claim 1, used as a screening program.
- 20. (Amended) A set of data relating to an influence on a cellular response in mechanically intact or permeabilised living cells, obtained by a method according to claim 1.

LRS/cqc

0459-0571P

REMARKS

The specification has been amended to provide a cross-reference to the previously filed International Application. The claims have also been amended to delete multiple dependencies and to place the application into better form for examination. Entry of the present amendment and favorable action on the above-identified application are earnestly solicited.

Attached hereto is a marked-up copy of the changes made to the application by this Amendment.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By MA Well 36,623 Leonard R. Svensson, #30,330

P.O. Box 747

Falls Church, VA 22040-0747

(703) 205-8000

Attachment: Version With Markings Showing Changes Made

(Rev. 01/22/01)

VERSION WITH MARKINGS SHOWING CHANGES MADE

The specification has been amended to provide cross-referencing to the International Application.

The claims have been amended as follows:

- 3. (Amended) A method according to claim 1 [or 2], wherein the influence comprises contact between the mechanically intact or permeabilised living cells and a chemical substance and/or incubation of the mechanically intact or permeabilised living cells with a chemical substance.
- 4. (Amended) A method according to [any of claims 1-3] <u>claim</u>

 1, wherein the cells comprise a group of cells contained within a spatial limitation.
- 5. (Amended) A method according to [any of claims 1-4] <u>claim</u>
 1, wherein the cells comprises multiple groups of cells contained within multiple spatial limitations.
- 6. (Amended) A method according to [any of claims 1-5] <u>claim</u>

 1, wherein the spatial limitations are spatial limitations arranged in one or more arrays on a common carrier.

- 8. (Amended) A method according to [any of claims 1-7] claim 1, wherein the redistribution results in quenching of fluorescence, the quenching being measure as a decrease in the intensity of the fluorescence.
- 9. (Amended) A method according to [any of claims 1-8] <u>claim</u>

 1, wherein the redistribution results in energy transfer, the energy transfer being measure as a change in the intensity of the luminescence.
- 10. (Amended) A method according to [any of claims 1-8] claim 1, wherein the intensity of the light being recorded is a function of the fluorescence lifetime, polarization, wavelength shift, or other property which is modulated as a result of the underlying cellular response.
- 11. (Amended) A method according to [any of claims 1-10] claim 1, wherein the light to be measured passes through a filter which selects the desired component of the light to be measured and rejects other components.
- 12. (Amended) A method according to [any of claims 1-11] claim 1, wherein the fluorescence comes from a fluorophore encoded by and expressed from a nucleotide sequence harboured in the cells.

- 13. (Amended) A method according to [any of the preceding claims] claim 1, wherein the fluorescence comes from a luminescent polypeptide, such as GFP.
- 14. (Amended) A method according to [any of the preceding claims] claim 1, wherein the luminescent polypeptide could be a GFP selected from the group consisting of green fluorescent proteins having the F64L such as F64L-GFP, F64L-Y66H-GFp, F64L-S65T-GFP, and EGFP.
- 15. (Amended) A method according to [any of claims 1-14] claim 1, wherein the cells are selected from the group consisting of fungal cells, such as yeast cells; invertebrate cells including insect cells; and vertebrate cells, such as mammalian cells.
- 17. (Amended) A method according to [any of claims 1-16] claim 1, used as a screening program.
- 20. (Amended) A set of data relating to an influence on a cellular response in mechanically intact or permeabilised living cells, obtained by a method according to [any of claims 1-19]claim 1.



BOX SEQUENCE PATENT 0459-0571P



IN THE U.S. PATENT AND TRADEMARK OFFICE

ARKHAMMAR, Per O.G. et al.

Conf.:

6261

Appl. No.:

09/807,345

Group:

Unassigned

Filed:

April 12, 2001

Examiner: Unassigned

For:

AN IMPROVED METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE ON A CELLULAR

RESPONSE

AMENDMENT

Assistant Commissioner for Patents Washington, DC 20231

August 14, 2001

Sir:

In response to the U.S. Patent Office Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Disclosures dated May 14, 2001, the period for response having been extended one (1) month to August 14, 2001, the following amendments and remarks are respectfully submitted in connection with the above-identified application.

IN THE SPECIFICATION:

Please replace the paragraph beginning on page 40, line 12 with the following amended paragraph:

-- To construct the PKAc-F64L-S65T-GFP fusion, convenient restriction endonuclease sites were introduced into the cDNAs encoding murine PKAc (Gen Bank Accession number: M12303) and F64L-S65T-GFP (sequence disclosed in

WO 97/11094) by polymerase chain reaction (PCR). The PCR reactions were performed according to standard protocols with the following primers:

5'PKAc:

TTggACACAAgCTTTggACACCCTCAggATATgggCAACgCCgCCgCCAAg (SEQ ID NO:19),

3'PKAc:

gTCATCTTCTCgAgTCTTTCAggCgCgCCCAAACTCAgTAAACTCCTTgCCACA C(SEQ ID NO:20)

5'GFP:

TTggACACAAgCTTTggACACggCgCgCCATgAgTAAAggAgAAACTTTT C(SEQ ID NO:21)

3'GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT (SEQ ID NO:22).--

Please replace the paragraph beginning on page 44, line 32 with the following amended paragraph:

--EXAMPLE 2 Probe for detection of PKC activity Construction of PKC-GFP fusion:

The probe was constructed by ligating two restriction enzyme treated polymerase chain reaction (PCR) amplification products of the cDNA for murine PKCα (GenBank Accession number: M25811) and F64L-S65T-GFP (sequence disclosed in WO 97/11094) respectively. Taq® polymerase and the following oligonucleotide primers were used for PCR;

5'mPKCa:

TTggACACAAgCTTTggACACCCTCAggATATggCTgACgTTTACCCggCCA ACg (SEQID NO:23)

3'mPKCa:

gTCATCTTCTCgAgTCTTTCAggCgCgCCCTACTgCACTTTgCAAgATTgggT gC (SEQ ID NO:24),

5'F64L-S65T-GFP:

TTggACACAAgCTTTggACACggCgCgCCCATgAgTAAAggAgAAACTTTT C (SEQ ID NO:25),

3'F64L-S65T-GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT (SEQ ID NO:26).--

Please replace the paragraph beginning on page 47, line 13 with the following amended paragraph:

--The human Erk1 gene (GenBank Accession number: X60188) was amplified using PCR according to standard protocols with primers

Erk1-top

5'-TAGAATTCAACCATGGCGGCGGCGGCGGCG (SEQ ID NO:27)-3' and Erk1-bottom/+stop
5'-TAGGATCCCTAGGGGGCCTCCAGCACTCC (SEQ ID NO:28)-3'.

The PCR product was digested with restriction enzymes EcoR1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with EcoR1 and BamH1. This produces an EGFP-Erk1 fusion (SEQ ID NOs: 5 and 6) under the control of a CMV promoter.--

Please replace the paragraph beginning on page 48, line 14 with the following amended paragraph:

- --Smad 2, a signal transducer, is a component of a signalling pathway that is induced by some members of the TGFbeta family of cytokines.
- a) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers Smad2-top
- 5'-GTGAATTCGACCATGTCGTCCATCTTGCCATTC (SEQ ID NO:29)-3' and Smad2-bottom/+stop
- 5'-GTGGTACCTTATGACATGCTTGAGCAACGCAC (SEQ ID NO:30)-3'.

 The PCR product was digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-C1 (Clontech; Palo Alto; GenBank Accession number U55763) digested with EcoR1 and Acc65I. This produces an EGFP-Smad2 fusion (SEQ ID NOs: 7 and 8) under the control of a CMV promoter.
- b) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers

 Smad2-top

5'-GTGAATTCGACCATGTCGTCCATCTTGCCATTC (SEQ ID NO:31)-3' and Smad2-bottom/-stop

5'-GTGGTACCCATGACATGCTTGAGCAACGCAC (SEQ ID NO:32)-3'.--

Please replace the paragraph beginning on page 49, line 16 with the following amended paragraph:

-- EXAMPLE 5 Probes for detection of VASP redistribution.

Useful for monitoring signalling pathways involving rearrangement of cytoskeletal elements, e.g. to identify compounds which modulate the activity of the pathway in living cells. VASP, a phosphoprotein, is a component of cytoskeletal structures, which redistributes in response to signals that affect focal adhesions.

The human VASP gene (GenBank Accession number: Z46389) was amplified using PCR according to standard protocols with primers

VASP-top

5'-GGGAAGCTTCCATGAGCGAGACGGTCATC (SEQ ID NO:33)-3' and VASP-bottom/+stop

5'-CCCGGATCCTCAGGGAGAACCCCGCTTC (SEQ ID NO:34)-3'.--

Please replace the paragraph beginning on page 50, line 4 with the following amended paragraph:

-- EXAMPLE 7 Probes for detection of NFkappaB redistribution.

Useful for monitoring signalling pathways leading to activation of NFkappaB, e.g. to identify compounds which modulate the activity of the pathway in living cells.

NFkappaB, an activator of transcription, is a component of signalling pathways that are responsive to a varity of inducers including cytokines, lymphokines, and some immunosuppressive agents.

- a) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers NFkappaB-top
- 5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC (SEQ ID NO:35)-3' and NFkappaB-bottom/+stop
- 5'-GTGGATCCTTAGGAGCTGATCTGACTCAGCAG (SEQ ID NO:36)-3'. The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Xho1 and BamH1. This produces an EGFP-NFkappaB fusion (SEQ ID NOs:13 and 14) under the control of a CMV promoter.
- b) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers NFkappaB-top
- 5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC (SEQ ID NO:37)-3'

and NFkappaB-bottom/-stop

5'-GTGGATCCAAGGAGCTGATCTGACTCAGCAG (SEQ ID NO:38)-3'.

The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-N1 (Clontech, Palo Alto; GenBank Accession number U55762) digested with Xho1 and BamH1. This produces an NFkappaB-EGFP fusion (SEQ ID NOs: 15 and 16) under the control of a CMV promoter.—

Please replace the paragraph beginning on page 54, line 1 with the following amended paragraph:

--EXAMPLE 11 Probes for detection of PKCβ1 redistribution.

Useful for monitoring signalling pathways involving Protein Kinase C, e.g. to identify compounds which modulate the activity of the pathway in living cells. PKCbeta1, a serine/threonine protein kinase, is closely related to PKCalpha and PKCbeta2 but not identical; it is a component of a signalling pathway which is activated by elevation of intracellular calcium concomitant with an increase in diacylglycerol species.

a) The human PKCbeta1 gene (GenBank Accession number: X06318) was amplified using PCR according to standard protocols with primers

PKCβ1-top

GTCTCGAGGCAAGATGGCTGACCC (SEQ ID NO:39)

and PKCβ1-bottom

GTGGATCCCTACACATTAATGACAAACTCTGGG (SEQ ID NO:40).--

Please replace the Sequence Listing filed April 12, 2001 located immediately after the claims with substitute Sequence Listing enclosed herewith.

REMARKS

Enclosed herewith in full compliance to 37 C.F.R. §§1.821-1.825 is a substitute Sequence Listing to be inserted into the specification as indicated above. The substitute Sequence Listing in no way introduces new matter into the specification.

Also submitted herewith in full compliance to 37 C.F.R. §§1.821-1.825 is a disk copy of the substitute Sequence Listing. The disk copy of the substitute Sequence Listing, file "0459-0571P.ST25", is identical to the paper copy, except that it lacks formatting.

The substitute Sequence Listing includes primer sequences found in the Specification as filed that were not made part of the original Sequence Listing. The amendments to the Specification are being made to reference these sequences by their SEQ ID NOS. These amendments are editorial in nature and do not constitute new matter.

Entry of the above amendments is earnestly solicited. An early and favorable first action on the merits is earnestly solicited.

Pursuant to C.F.R. §§1.17 and 1.136(a), the Applicant respectfully petitions for a one (1) month extension of time for filing a response in connection with the present application and the required fee of \$110.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

Leonard R. Svensson, #30,330

LRS/KW 0459-0571P P.O. Box 747 Falls Church, VA 22040-0747 (703) 205-8000

Attachments: Paper and disk copy of Sequence Listing

Copy of Notice to Comply

Copy of Version with Markings to Show Changes Made

VERSION WITH MARKING TO SHOW CHANGES MADE

The paragraph beginning on page 40, line 12 has been amended as follows:

To construct the PKAc-F64L-S65T-GFP fusion, convenient restriction endonuclease sites were introduced into the cDNAs encoding murine PKAc (Gen Bank Accession number: M12303) and F64L-S65T-GFP (sequence disclosed in WO 97/11094) by polymerase chain reaction (PCR). The PCR reactions were performed according to standard protocols with the following primers:

5'PKAc:

 $TTggACACAAgCTTTggACACCCTCAggATATgggCAACgCCgCCgCCgCCAAg\underline{\ (SEQ\ ID\ Carrow Constraints)}$

NO:19),

3'PKAc:

 ${\tt gTCATCTTCTCgAgTCTTTCAggCgCgCCCAAACTCAgTAAACTCCTTgCCACAC} ({\tt SEQ\,ID}$

NO:20)

5'GFP:

 $TTggACACAAgCTTTggACACggCgCCCATgAgTAAAggAgAAGAACTTTTC \underline{(SEQ\ ID\ ACACAAgCTTTTC)}$

NO:21)

3'GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT($\underline{SEQ\ ID}$ NO:22).

The paragraph beginning on page 44, line 32 has been amended as follows:

EXAMPLE 2 Probe for detection of PKC activity

Construction of PKC-GFP fusion:

The probe was constructed by ligating two restriction enzyme treated polymerase chain reaction (PCR) amplification products of the cDNA for murine PKCα (GenBank Accession number:

M25811) and F64L-S65T-GFP (sequence disclosed in WO 97/11094) respectively. Taq® polymerase and the following oligonucleotide primers were used for PCR;

5'mPKCα: TTggACACAAgCTTTggACACCCTCAggATATggCTgACgTTTACCCggCCAACg (SEQID NO:23)

3'mPKCa:

gTCATCTTCTCgAgTCTTTCAggCgCgCCCTACTgCACTTTgCAAgATTgggTgC (SEQ ID NO:24),

5'F64L-S65T-GFP:

TTggACACAAgCTTTggACACggCgCgCCATgAgTAAAggAgAAACTTTTC (SEQ ID NO:25),

3'F64L-S65T-GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT (SEQ ID NO:26).

The paragraph beginning on page 47, line 13 has been amended as follows:

The human Erk1 gene (GenBank Accession number: X60188) was amplified using PCR according to standard protocols with primers

Erk1-top

5'-TAGGATCCCTAGGGGGCCTCCAGCACTCC (SEQ ID NO:28)-3'.

The PCR product was digested with restriction enzymes EcoR1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with EcoR1 and

BamH1. This produces an EGFP-Erk1 fusion (SEQ ID NOs: 5 and 6) under the control of a CMV promoter.

The paragraph beginning on page 49, line 16 has been amended as follows:

Smad 2, a signal transducer, is a component of a signalling pathway that is induced by some members of the TGFbeta family of cytokines.

a) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers

Smad2-top

5'-GTGAATTCGACCATGTCGTCCATCTTGCCATTC (SEQ ID NO:29)-3' and Smad2-bottom/+stop

5'-GTGGTACCTTATGACATGCTTGAGCAACGCAC (SEQ ID NO:30)-3'.

The PCR product was digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-C1 (Clontech; Palo Alto; GenBank Accession number U55763) digested with EcoR1 and Acc65I. This produces an EGFP-Smad2 fusion (SEQ ID NOs: 7 and 8) under the control of a CMV promoter.

- b) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers

 Smad2-top
- 5'-GTGAATTCGACCATGTCGTCCATCTTGCCATTC (SEQ ID NO:31)-3' and Smad2-bottom/-stop
- 5'-GTGGTACCCATGACATGCTTGAGCAACGCAC (SEQ ID NO:32)-3'.

The paragraph beginning on page 48, line 14 has been amended as follows:

EXAMPLE 5 Probes for detection of VASP redistribution.

Useful for monitoring signalling pathways involving rearrangement of cytoskeletal elements, e.g. to identify compounds which modulate the activity of the pathway in living cells. VASP, a phosphoprotein, is a component of cytoskeletal structures, which redistributes in response to signals that affect focal adhesions.

The human VASP gene (GenBank Accession number: Z46389) was amplified using PCR according to standard protocols with primers

VASP-top

5'-GGGAAGCTTCCATGAGCGAGACGGTCATC (SEQ ID NO:33)-3' and VASP-bottom/+stop

5'-CCCGGATCCTCAGGGAGAACCCCGCTTC (SEQ ID NO:34)-3'.

The paragraph beginning on page 50, line 4 has been amended as follows:

EXAMPLE 7 Probes for detection of NFkappaB redistribution.

Useful for monitoring signalling pathways leading to activation of NFkappaB, e.g. to identify compounds which modulate the activity of the pathway in living cells.

NFkappaB, an activator of transcription, is a component of signalling pathways that are responsive to a varity of inducers including cytokines, lymphokines, and some immunosuppressive agents.

a) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers

NFkappaB-top

5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC (SEQ ID NO:35)-3'

and NFkappaB-bottom/+stop

5'-GTGGATCCTTAGGAGCTGATCTGACTCAGCAG (SEQ ID NO:36)-3'.

The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Xho1 and BamH1. This produces an EGFP-NFkappaB fusion (SEQ ID NOs:13 and 14) under the control of a CMV promoter.

b) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers

NFkappaB-top

5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC (SEQ ID NO:37)-3'

and NFkappaB-bottom/-stop

5'-GTGGATCCAAGGAGCTGATCTGACTCAGCAG (SEQ ID NO:38)-3'.

The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-N1 (Clontech, Palo Alto; GenBank Accession number U55762) digested with Xho1 and BamH1. This produces an NFkappaB-EGFP fusion (SEQ ID NOs: 15 and 16) under the control of a CMV promoter.

The paragraph beginning on page 54, line 1 has been amended as follows:

EXAMPLE 11 Probes for detection of PKC β 1 redistribution.

Useful for monitoring signalling pathways involving Protein Kinase C, e.g. to identify compounds which modulate the activity of the pathway in living cells.

PKCbeta1, a serine/threonine protein kinase, is closely related to PKCalpha and PKCbeta2 but not identical; it is a component of a signalling pathway which is activated by elevation of intracellular calcium concomitant with an increase in diacylglycerol species.

a) The human PKCbeta1 gene (GenBank Accession number: X06318) was amplified using PCR according to standard protocols with primers

PKCβ1-top

GTCTCGAGGCAAGATGGCTGACCC (SEQ ID NO:39)

and PKCβ1-bottom

 ${\tt GTGGATCCCTACACATTAATGACAAACTCTGGG} \underline{({\tt SEQ~ID~NO:40})}.$

WO 00/23615

PCT/DK99/00562

AN IMPROVED METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE ON A CELLULAR RESPONSE.

SUMMARY OF THE INVENTION

5 The present invention relates to an improved method and tools for extracting quantitative information relating to an influence on a cellular response, in particular an influence caused by contacting or incubating the cell with a substance influencing a cellular response, wherein the cellular response is manifested in redistribution of at least one component in the cell. In particular, the invention relates to an improved method for 10 extracting the quantitative information relating to an influence on an intracellular pathway involving redistribution of at least one component associated with the pathway. The method of the invention may be used as a very efficient procedure for testing or discovering the influence of a substance on a physiological process, for example in connection with screening for new drugs, testing of substances for toxicity, identifying 15 drug targets for known or novel drugs. In particular, the present invention relates to an improved method for parallelisation of the testing procedure so that a large number of substances can be tested simultaneously using commercially available instrumentation. The invention also describes several ways of contacting the cells with a substance influencing a cellular response and modifications made to the actual cells before, during 20 or after contacting the cells with these substances as to improve the applicability and use of the method for extracting quantitative information relating to influence on an intracellular pathway in a highly parallel fashion. Other valuable uses of the method and technology of the invention will be apparent to the skilled person on the basis of the following disclosure. In a particular embodiment of the invention, the present invention 25 relates to a method of detecting intracellular translocation or redistribution of biologically active polypeptides, preferably an enzyme, affecting intracellular processes, and a DNA

BACKGROUND OF THE INVENTION

construct and a cell for use in the method.

30 Intracellular pathways are tightly regulated by a cascade of components that undergo modulation in a temporally and spatially characteristic manner. Several disease states can be attributed to altered activity of individual signalling components (i.e. protein

kinases, protein phosphatases, transcription factors). These components therefore render themselves as attractive targets for therapeutic intervention.

2

Protein kinases and phosphatases are well-described components of several

intracellular signalling pathways. The catalytic activity of protein kinases and phosphatases are assumed to play a role in virtually all regulatable cellular processes. Although the involvement of protein kinases in cellular signalling and regulation have been subjected to extensive studies, detailed knowledge on e.g. the exact timing and spatial characteristics of signalling events is often difficult to obtain due to lack of a convenient technology.

The measurement of the activity of intracellular enzymes, such as kinases and phosphatases, can be performed by well-established procedures, both manually and in various automated forms, at throughput rates which make these measurements useful in the search for new drug candidates. In addition to measures of activity, measures of the distribution of these and other enzymes in the cell has proven useful, and established techniques exist for this type of measurement as well. Protein kinases often show a specific intracellular distribution before, during and after activation. Monitoring the translocation processes and/or redistribution of individual protein kinases or subunits thereof is thus likely to be indicative of their functional activity. A connection between translocation and catalytic activation has been shown for protein kinases like the diacyl glycerol (DAG)-dependent protein kinase C (PKC), the cAMP-dependent protein kinase (PKA) [(DeBernardi et al.1996)] and the mitogen-activated-protein kinase Erk-1 [(Sano et al.1995)]. Such methods of detection of intracellular localisation/activity of protein kinases and phosphatases include immunoprecipitation, Western blotting and immunocytochemical detection.

One aspect of the function of intracellular enzymes which has not been characterised so thoroughly is the redistribution of those enzymes. The importance of subcellular redistribution of enzymes as a mechanism of enzyme specificity, and of the general importance of the measurement of subcellular redistribution as a tool for identifying novel drug targets and searching for drug candidates which influence those targets, is disclosed in: A METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE ON A CELLULAR RESPONSE the contents of which

were part of the priority application, and which, as WO9845704 has been published during the priority year, are hereby incorporated herein by reference.

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While the redistribution of subcellular components is known to be important, the

measurement of this phenomenon in real time has not been widely exploited. This is
primarily due to the lack of a suitable technique. There is essentially only one direct
technique: the microscopic imaging of cells in which the subcellular component of
interest has been labelled in such a way that it can be visualised and recorded by the
microscopic imaging system, using for example a video or scientific CCD camera and
appropriate software for collecting and storing the images. Novel ways of monitoring
specific modulation of intracellular pathways in intact, living cells is assumed to provide
new opportunities in drug discovery, functional genomics, toxicology, patient monitoring
etc.

15 Recently it was discovered that Green Fluorescent Protein (GFP) expressed in many different cell types, including mammalian cells, became highly fluorescent [(Chalfie et al. 1994)]. WO95/07463 describes a cell capable of expressing GFP and a method for detecting a protein of interest in a cell based on introducing into a cell a DNA molecule having DNA sequence encoding the protein of interest linked to DNA sequence encoding 20 a GFP such that the protein produced by the DNA molecule will have the protein of interest fused to the GFP, then culturing the cells in conditions permitting expression of the fused protein and detecting the location of the fluorescence in the cell, thereby localizing the protein of interest in the cell. However, examples of such fused proteins are not provided, and the use of fusion proteins with GFP for detection or quantitation of 25 translocation or redistribution of biologically active polypeptides affecting intracellular processes upon activation, such as proteins involved in signalling pathways, e.g. protein kinases or phosphatases, has not been suggested. WO 95/07463 further describes cells useful for the detection of molecules, such as hormones or heavy metals, in a biological sample, by operatively linking a regulatory element of the gene which is affected by the 30 molecule of interest to a GFP, the presence of the molecules will affect the regulatory element which in turn will affect the expression of the GFP. In this way the gene encoding GFP is used as a reporter gene in a cell which is constructed for monitoring the presence of a specific molecular identity.

Green Fluorescent Protein has been used in an assay for the detection of translocation of the glucocorticoid receptor (GR) [(Carey, KL et al. 1996)]. A GR-S65TGFP fusion has been used to study the mechanisms involved in translocation of the glucocorticoid receptor (GR) in response to the agonist dexamethasone from the cytosol, where it is 5 present in the absence of a ligand, through the nuclear pore to the nucleus where it remains after ligand binding. The use of a GR-GFP fusion enables real-time imaging and quantitation of nuclear/cytoplasmic ratios of the fluorescence signal. A similar genetic construct has been used to follow and quantify dexamethasone induced translocation of GR to the nucleus in HeLa cells [(Guiliano, K.A et al. 1997)] in a system called Array 10 Scan™ (WO 97/45730) designed for automated drug screening. Recently, several other investigators have demonstrated that tagging a specific protein (or part of a protein) involved in an intracellular signalling pathway with GFP provides a new means to measure and quantify the influence of substances on this pathway. The concept has been shown to work both for cytoplasmic to nuclear translocation of the androgen 15 receptor [(Georget V et al. 1997)] and transcription factors such as NF-ATc [(Beals CR et al. 1997)] in analogy with what has already been described for GR above. Another relevant example is a β -arrestin – GFP construct that was shown to report on activation of G-protein coupled receptors by translocating from the cytosol to the plasma membrane [(Barak LS et al. 1997)]. Finally, it has also been demonstrated that attaching 20 GFP to a smaller part of a protein like the pleckstrin homology domain of phospholipase C δ 1 [(Stauffer TP et al. 1998)] and a cysteine-rich domain of PKC γ [(Oancea E et al. 1998)] can be used to report on an influence from a substance by quantifying their redistribution within the cells during activation of the specific signalling pathway to which they belong.

25

Many currently used screening programmes designed to find compounds that affect protein kinase activity are based on measurements of kinase phosphorylation of artificial or natural substrates, receptor binding and/or reporter gene expression. The interest in fluorescence measurements as the basis for future high-throughput drug screening has however increased dramatically over the last few years [(Silverman L *et al.* 1998)]. Of particular interest to the present invention is a scanning laser imager for rapid screening of fluorescence changes in living cells [(Schroeder K & Neagle B 1996)] currently offered commercially by Molecular Devices, Inc. as the FLIPR™.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an important new dimension in the investigation of cellular systems involving redistribution in that the invention provides quantification of the redistribution responses or events caused by an influence, typically contact with a chemical substance or mixture of chemical substances, but also changes in the physical environment, in a massively parallel fashion. The quantification makes it possible to set up meaningful relationships, expressed numerically, or as curves or graphs, between the influences (or the degree of influences) on cellular systems and the redistribution

10 response. This is highly advantageous because, as has been found, the quantification can be achieved in both a fast and reproducible manner, and - what is perhaps even more important - the systems which become quantifiable utilising the method of the invention are systems from which enormous amounts of new information and insight can be derived.

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The present screening assays have the distinct advantage over other screening assays, e.g., receptor binding assays, enzymatic assays, and reporter gene assays, in providing a system in which biologically active substances with completely novel modes of action, e.g. inhibition or promotion of redistribution/translocation of a biologically active polypeptide as a way of regulating its action rather than inhibition/activation of enzymatic activity, can be identified in a way that insures very high selectivity to the particular isoform of the biologically active polypeptide and further development of compound selectivity versus other isoforms of the same biologically active polypeptide or other components of the same signalling pathway.

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In one of its broadest aspects, the invention relates to an improved method, with higher throughput compared to previous methods, for extracting quantitative information relating to an influence on a cellular response, the method comprising recording variation, caused by the influence on mechanically intact living cells, in spatially distributed light emitted from a luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence, the association resulting in a modulation of the luminescence characteristics of the luminophore,

35 detecting and recording the variation in spatially distributed light from the luminophore as

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a change in fluorescence intensity using an instrument designed to measure changes in fluorescence intensity, and processing the recorded variation in the spatially distributed light to provide quantitative information correlating the spatial distribution or change in the spatial distribution to the degree of the influence. In one aspect of the present 5 invention the mechanically intact living cell is permeabilised at some time after the influence has begun but during or before the actual experimental recording. In another aspect, the present invention relates to an improved method for extracting quantitative information relating to an influence on a cellular response, the method comprising recording variation, caused by the influence on permeabilised living cells, in spatially 10 distributed light emitted from a luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence, the association resulting in a modulation of the luminescence characteristics of the luminophore, 15 detecting and recording the spatially distributed light from the luminophore as a change in fluorescence intensity using an instrument designed to measure changes in fluorescence intensity, and processing the recorded variation in the spatially distributed light to provide quantitative information correlating the spatial distribution or change in the spatial distribution to the degree of the influence. In a preferred embodiment of the 20 invention the luminophore, which is present in the cells, is capable of being redistributed by modulation of an intracellular pathway, in a manner which is related to the redistribution of at least one component of the intracellular pathway. In another preferred embodiment of the invention, the luminophore is a fluorophore.

25 Typically the cell and/or cells are mechanically intact and alive throughout the experiment. In another embodiment of the invention, the cells are fixed at a point in time after the application of the influence at which the response has been predetermined to be significant, and the recording is made at an arbitrary later time. In another embodiment the cell and/or cells are mechanically intact and alive throughout the experiment but are mechanically or chemically disrupted or permeabilised as the initial step of experimental analysis. In another aspect of the invention the cells have their plasma membrane permanently and stably permeabilised before the initiation of the experiment in such a way that the plasma membrane stays permeable during the experiment. This allows the components of intracellular pathways to be contacted by

substances that are not normally permeating the cell plasma membrane such as peptides, proteins and hydrophilic organic compounds..

The mechanically intact or permeabilised living cells could be selected from the group consisting of fungal cells, such as yeast cells; invertebrate cells including insect cells; and vertebrate cells, such as mammalian cells. These cells are incubated at a temperature of 30°C or above, preferably at a temperature of from 32°C to 39°C, more preferably at a temperature of from 35°C to 38°C, and most preferably at a temperature of about 37°C during the time period over which the influence is observed. In one aspect of the invention the mechanically intact or permeabilised living cell is part of a matrix of identical or non-identical cells. In one embodiment of the invention the cells comprise a group or groups of cells contained within a spatial limitation or spatial limitations. In one embodiment, the cells comprise multiple groups of cells that are qualitatively the same but subjected to different influences. In another embodiment, the cells comprise multiple groups of cells that are qualitatively different but subjected to the same influence.

In one embodiment of the invention the spatial limitations are domains defined on a substrate on which the cells are present. The spatial limitations may be arranged in one or more arrays on a common carrier. The spatial limitations may be wells in a plate of 20 microtiter type, such that 96, 384. 864 and 1536 wells are situated on the common carrier. In another embodiment the spatial limitations are wells in a plate of a format different from the microtiter type. In one embodiment of the invention the domains are established by the presence of the cells on the substrate in a pattern that defines the domains. In another aspect of the invention, the domains are instead established by the 25 spatial pattern or array of the influence or influences as it/they are applied to or contacted by the cells. This aspect is thoroughly disclosed in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which. as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference. Briefly, in this aspect of the 30 invention the mechanically intact or permeabilised living cells are part of a continuous or discontinuous sheet of cells cultured on an optically clear flat surface typically optimised for cell culture. The optically clear and flat surface may be a porous membrane that may allow cellular processes to grow through the membrane pores and may allow directed capillary flow of fluid through the pores.

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A cell used in the present invention should contain a nucleic acid construct encoding a fusion polypeptide as defined herein and be capable of expressing the sequence encoded by the construct. The cell is a eukaryotic cell selected from the group consisting of fungal cells, such as yeast cells: invertebrate cells including insect cells; vertebrate cells such as mammalian cells. The preferred cells are mammalian cells.

In another aspect of the invention the cells could be from an organism carrying in at least one of its component cells a nucleic acid sequence encoding a fusion polypeptide as defined herein and be capable of expressing said nucleic acid sequence. The organism is selected from the group consisting of unicellular and multicellular organisms, such as a mammal.

The luminophore is the component that allows the redistribution to be visualised and/or recorded by emitting light in a spatial distribution related to the degree of influence. The 15 term redistribution is intended to cover all aspects of a change in spatial location, such as a translocation of the luminophore or other components. In one embodiment of the invention, the luminophore is capable of being redistributed in a manner that is physiologically relevant to the degree of the influence. In another embodiment, the luminophore is capable of associating with a component that is capable of being 20 redistributed in a manner that is physiologically relevant to the degree of the influence. In another embodiment, a correlation between the redistribution of the luminophore and the degree of the influence could be determined experimentally. In a preferred aspect of the invention, the luminophore is capable of being redistributed in substantially the same manner as the at least one component of an intracellular pathway. In another 25 embodiment of the invention, the luminophore is capable of being quenched upon spatial association with a component that is redistributed by modulation of the pathway, the quenching being measured as a change in the intensity of the luminescence. In another embodiment of the invention, the luminophore is stationary but may have a certain spatial distribution, and interacts with at least one component that is capable of being 30 redistributed in a manner which is physiologically relevant to the degree of the influence, in such a way that one or more luminescence characteristics of the luminophore is/are modulated as the component moves closer to, or farther from, the luminophore.

The luminophore could be a fluorophore. In a preferred embodiment of the invention, the luminophore is a polypeptide encoded by and expressed from a nucleotide sequence

harboured in the cells. The luminophore could be a hybrid polypeptide comprising a fusion of at least a portion of each of two polypeptides one of which comprises a luminescent polypeptide and the other one of which comprises a biologically active polypeptide, as defined herein.

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The luminescent polypeptide could be a GFP as defined herein or could be selected from the group consisting of green fluorescent proteins having the F64L mutation as defined herein such as F64L-GFP, F64L-Y66H-GFP, F64L-S65T-GFP, and EGFP. The GFP could be N- or C-terminally tagged, optionally via a peptide linker, to the biologically active polypeptide or a part or a subunit thereof. The fluorescent probe could be a component of an intracellular signalling pathway. The probe is coded for by a nucleic acid construct.

In one aspect of the invention the pathway of investigation is an intracellular signalling pathway.

In a preferred embodiment of the invention, the influence could be contact between the group or groups of mechanically intact or permeabilised living cells and a chemical substance, and/or incubation of the group or groups of mechanically intact or permeabilised living cells with a chemical substance in solution. In one aspect of the invention that is thoroughly described in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference, the chemical substances are attached to an underlying matrix. In this aspect, the chemical substances may also be produced and secreted from, or attached to the plasma membrane surfaces of, a sheet of genetically engineered cells. In this aspect of the invention the chemical substances may also have been separated two-dimensionally in a non-denaturing gel using electrophoresis and the gel is directly put in close proximity or direct contact with the mechanically intact or permeabilised living cells so that the chemical substances can contact the cells through diffusion or convection.

The influence will modulate the intracellular processes. In one aspect the modulation could be an activation of the intracellular processes. In another aspect the modulation could be a deactivation of the intracellular processes. In yet another aspect, the

influence could inhibit or promote the redistribution without directly affecting the metabolic activity of the component of the intracellular processes.

In one embodiment the invention is used to establish a dose-response relationship for one or many chemical substances. In one embodiment the invention is used as a basis for a screening program, where the effect of unknown influences such as a compound library, can be compared to influence of known reference compounds under standardised conditions.

- In addition to the intensity, there are several parameters of fluorescence or luminescence that can be modulated by the effect of the influence on the underlying cellular phenomena, and can therefore be used in the invention. Some examples are resonance energy transfer, fluorescence lifetime, polarisation, and wavelength shift. Each of these methods requires a particular kind of filter in the emission light path to select the
 component of the light desired and reject other components. The recording of property of light could be in the form of an ordered array of values such as a CCD array or a vacuum tube device such as a vidicon. In addition, the translational mobility, or freedom of movement, of the luminophore attached to the protein of interest can be an important property affected by the influence on the underlying cellular phenomena, and can
 therefore be used in the invention.
- In one embodiment of the invention, the spatially distributed light emitted by a luminophore is detected by a change in the resonance energy transfer between the luminophore and another luminescent entity capable of delivering energy to the luminophore, each of which has been selected or engineered to become part of, bound to or associated with particular components of the intracellular pathway. In this embodiment, either the luminophore or the luminescent entity capable of delivering energy to the luminophore undergoes redistribution in response to an influence. The resonance energy transfer would be measured as a change in the intensity of emission from the luminophore, preferably sensed by a single channel photodetector that responds only to the average intensity of the luminophore in a non-spatially resolved fashion.

In one embodiment of the invention, the spatially distributed light emitted by a 35 luminophore includes the case of uniform spatial distribution of the light.

In one aspect of the invention, the luminophore is a fluorophore which redistributes through a non-homogenous excitation light field, resulting in a change in the intensity of the light emitted from the luminophore as a result of the change in the amount of excitation light intensity at different points in the field.

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In one embodiment of the invention, the recording of the spatially distributed light could be made at a single point in time after the application of the influence. In another embodiment, the recording could be made at two points in time, one point being before, and the other point being after the application of the influence. The result or variation is determined from the change in fluorescence compared to the fluorescence measured prior to the influence or modulation. In another embodiment of the invention, the recording could be performed at a series of points in time, in which the application of the influence occurs at some time after the first time point in the series of recordings, the recording being performed, e.g., with a predetermined time spacing of from 0.1 seconds to 1 hour, preferably from 1 to 60 seconds, more preferably from 1 to 30 seconds, in particular from 1 to 10 seconds, over a time span of from 1 second to 12 hours, such as from 10 seconds to 12 hours, e.g., from 10 seconds to one hour, such as from 60 seconds to 30 minutes or 20 minutes. The result or variation is determined from the change in fluorescence over time. The result or variation could also be determined as a change in the spatial distribution of the fluorescence over time.

In one embodiment the recording comprises a time series of total luminescence of the cells of one or several of the spatial limitations. In one embodiment the signal from all of the spatial limitations, one at a time, is measured by a recording being made in the individual spatial limitations by means of an apparatus to sequentially position each one of the limitations in the field of view of the detector and repeating the positioning and measurement process until all of the spatial limitations have been measured. The detector may be a photomultiplier tube. In a preferred embodiment of the present invention more than one spatial limitation is measured simultaneously. This may be done by means of a one- or two-dimensional array detector, whereby the multiple spatial limitations are imaged onto the array detector such that discrete subsets of the detecting units (pixels) in the array detector measure the signal from one and only one of the multiple spatial limitations, the signal from any one spatial limitation being the combined signal from those pixels that receive the image from one of the spatial limitations. This

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array detector may be a linear diode array, a video camera (according to any present or future standards and definitions of image acquisition and transmission) or a charge transfer device such as a charge-coupled device (CCD). In one embodiment the recording of signal requires illumination of the multiple spatial limitations to excite the luminophores so that they emit light. In one embodiment all of the spatial limitations are simultaneously illuminated during the measurement. In another embodiment the spatial limitations are singly illuminated only during the time in which they are being measured. In a preferred embodiment the illumination is provided by a laser that is scanned in a raster fashion over some or all of the spatial limitations being measured. The scanning may take place at a rate that is substantially faster than the measurement process such that the illumination appears to the measurement process to be continuous in time and spatially uniform over the region being measured.

The recording of spatially distributed luminescence emitted from the luminophore is

performed by an apparatus for measuring the distribution of fluorescence in the cells,
and thereby any change in the distribution of fluorescence in the cells, which includes at
a minimum the following component parts: (a) a light source, (b) a method for selecting
the wavelength(s) of light from the source which will excite the luminescence of the
luminophore, (c) a device which can rapidly block or pass the excitation light into the rest
of the system, (d) a series of optical elements for conveying the excitation light to the
specimen, collecting the emitted fluorescence in a spatially resolved fashion, and forming
an image from this fluorescence emission (or another type of intensity map relevant to
the method of detection and measurement), (e) a bench or stand which holds the
container of the cells being measured in a predetermined geometry with respect to the
series of optical elements, (f) a detector to record the spatially resolved fluorescence in
the form of an image, (g) a computer or electronic system and associated software to
acquire and store the recorded images, and to compute the degree of redistribution from
the recorded images.

30 In a preferred embodiment of the invention the apparatus system is automated. In one embodiment the components in d and e mentioned above comprise a fluorescence microscope. In one embodiment the component in f mentioned above is a CCD camera. In one embodiment the component in f mentioned above is an array of photomultiplier tubes/devices.

In one embodiment the image is formed and recorded by an optical scanning system.

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In one embodiment the optical scanning system is used to illuminate the bottom of a plate of microtiter type so that a time-resolved recording of changes in luminescence or fluorescence can be made from all spatial limitations simultaneously.

In a preferred embodiment the actual luminescence or fluorescence measurements are made in a FLIPR™ instrument, commercially available from Molecular Devices, Inc.

- 10 In one embodiment of the invention the actual fluorescence measurements are made in a standard type of fluorometer for plates of microtiter type (fluorescence plate reader).
 - In one embodiment a liquid addition system is used to add a known or unknown compound to any or all of the cells in the cell holder at a time determined in advance.
- 15 Preferably, the liquid addition system is under the control of the computer or electronic system. Such an automated system can be used for a screening program due to its ability to generate results from a larger number of test compounds than a human operator could generate using the apparatus in a manual fashion.
- 20 The methods whereby the detector layer of cells are physically contacted by the compounds can also be of another conceptual type where the compounds are delivered to the cells through a porous membrane by convection/diffusion or by directly contacting compounds attached to an inorganic or organic support (such as glass, plastic or the plasma membrane of intact living cells) with the cells. These methods are thoroughly
- described in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference, but are also outlined in the following paragraphs.
- 30 In one aspect of the present invention where the detector layer of cells is a continuous or discontinuous sheet of cells without any separation into test units or wells. The compounds are printed onto a nonabsorbent sheet of porous material as a solution in solvent and allowed to dry. This printed sheet of compounds then defines the test pattern for the experiment as it is brought down in close proximity to or in direct contact with the underlying detector layer of cells. The compounds, now dissolved by the fluid layer on

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the cells, is brought in contact with the cells through the pores of the membrane by convection. The porous membrane onto which the compounds are printed is optically clear and preferably composed as stated in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority 5 application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference. In another embodiment of this aspect of the present invention the detector layer of cells is a continuous or discontinuous sheet of cells, without any separation into test units or wells, growing on a porous and optically clear membrane preferably of the types mentioned above. The porous membrane may 10 allow the cells to send cellular processes through the pores of the membrane. The compounds are printed onto an optically clear substratum such as glass, plastic or quartz as solutions in solvent and allowed to dry. At the time of the experiment the cell sheet on the membrane, surrounded by a thin film of fluid, is layered ontop of the printed compound pattern. The compounds then dissolve and contact the cells via diffusion and 15 convection. The compounds may be made using combinatorial chemistry techniques, and may be peptides. The compounds may be covalently attached to the optically clear substratum or porous membrane. The compounds may also be proteins, polypeptides or peptides secreted by or attached to the plasma membrane of genetically modified cells growing as a continuous or discontinuous sheet on a flat optically clear surface or an 20 optically clear porous membrane.

The recording of the variation or result with respect to light emitted from the luminophore is performed by recording the spatially distributed light as one or more digital images, and the processing of the recorded variation to reduce it to one or more numbers representative of the degree of redistribution comprises a digital image processing procedure or combination of digital image processing procedures. The quantitative information which is indicative of the degree of the cellular response to the influence or the result of the influence on the intracellular pathway is extracted from the recording or recordings according to a predetermined calibration based on responses or results, recorded in the same manner, to known degrees of a relevant specific influence. This calibration procedure is developed according to principles described below (Developing an Image-based Assay Technique). Specific descriptions of the procedures for particular assays are given in the examples.

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While the stepwise procedure necessary to reduce the image or images to the value representative of the response caused by the influence is particular to each assay, the individual steps are generally well-known methods of image processing. Some examples of the individual steps are point operations such as subtraction, ratioing, and

5 thresholding, digital filtering methods such as smoothing, sharpening, and edge detection, spatial frequency methods such as Fourier filtering, image cross-correlation and image autocorrelation, object finding and classification (blob analysis), and colour space manipulations for visualisation. In addition to the algorithmic procedures, heuristic methods such as neural networks may also be used. In a preferred embodiment of the

10 invention, a dose-response relationship is established based on quantification of the responses caused by a particular influence, representative of the underlying intracellular signalling process, using the methods described above and in the examples. The dose-response relationship for the particular influence is then compared to the dose-response relationship obtained by performing the same assay in an instrument which allows

parallel monitoring of all wells in a microtiter plate such as a FLIPR™ or an ordinary fluorescence plate reader for microtiter plates. If a good correlation between the dose-response relationships obtained from the two different measurement systems is obtained, it can be said that the parallel measurement mode has been validated (see examples 8, 9, 10 and 11). This implies that it can be used as the primary basis for a screening assay with the potential benefit of screening a significantly higher number of

substances per unit of time for their influence on the response. For example, if the single experiment performed on the microscope can be run in at least 96 experimental chambers simultaneously the throughput for the person who is running the experiments increases by a factor of 96.

Imaging plate readers integrate the signal from each well into a single value per time point. Thus the data resulting from a single "run" of the instrument is a set of time series of single values, one for each well, with the injection of the test compound taking place at a known point in the time series. The primary advantage of this type of instrumentation is that it greatly increases the number of samples that can be processed in a given amount

of time (the throughput). This is of great advantage when using the assay in a screening program for new pharmaceutical lead compounds.

The first step in the data analysis is to normalise the results from each well so that they can be compared with each other or with previously analysed known compounds. This

always begins with correcting the signal by subtracting the instrument bias from all data points on a well-by-well basis. From this point, either of two techniques can be followed depending on the design of the assay:

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Procedure 1: The average of the signal prior to the addition of the test compound is subtracted from all data points on a well-by-well basis.

Procedure 2: The data are corrected for any known background by subtracting the background value from all data points on a well-by-well basis. The resulting background-corrected data are normalised by dividing each data set by the average of the data values prior to the injection of the test compound on a well-by-well basis.

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The corrected or normalised time series data sets are then further reduced by a technique that converts the time series to a single value. There are at least three such approaches:

For transient responses, the maximum deviation from the baseline is determined. This is also known as the "peak height" technique.

Alternatively, the signal is integrated over time between pre-defined limits. If the data were treated according to Procedure 2 above, then the offset is subtracted such that the integral of a non-response is zero within the limit of measurement error. This is also known as the "peak area" technique. If the response is a cumulative one, e.g., an exponential change to a new level, the result is taken as the either the difference or the ratio between the signal after a predetermined time and the signal prior to the addition of the test compound.

25 All of the above procedures reduce the data for a given well to one or more single values. For screening purposes, these values will be searched for those that are greater than a certain statistically determined cut-off value. For characterisation, the values represent a quantitative response, and are further treated in sets by techniques such as dose-response curve fitting.

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In another embodiment of the invention, the measurement of redistribution is accomplished indirectly by taking advantage of the fact that in order for redistribution to occur, the probe will experience some change in its freedom, or restriction, of movement within the intracellular milieu. The degree of translocation will correlate with the amount of freely mobile luminophore in the cytoplasm. At a point in time after the test compound

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has begun to have any influence it may have, the amount or fraction of restricted luminophore can be measured by disrupting or permeabilising the plasma membrane of the cells and allowing the freely mobile luminophore to diffuse away. If the detection volume of the detector is limited to the region immediately surrounding the cells, and the overall volume into which the freely mobile luminophore can diffuse is much larger, then the freely mobile luminophore essentially disappears from the detector's view and its signal is not recorded.

In one aspect of the invention, the above mentioned measurement of redistribution is

made on cells with permanently permeabilised plasma membranes immersed in a
solution mimicking the cytoplasmic environment. In this way the influence of compounds
that can normally not enter the cytoplasm of cells can be tested.

The nucleic acid constructs used in the present invention encode in their nucleic acid sequences fusion polypeptides comprising a biologically active polypeptide that is a component of an intracellular signalling pathway, or a part thereof, and a GFP, preferably an F64L mutant of GFP, N- or C-terminally fused, optionally via a peptide linker, to the biologically active polypeptide or part thereof. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein kinase or a phosphatase. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a transcription factor or a part thereof which changes cellular localisation upon activation.

In one embodiment the biologically active polypeptide encoded by the nucleic acid
construct is a protein, or a part thereof, which is associated with the cytoskeletal network
and which changes cellular localisation upon activation. In one embodiment the
biologically active polypeptide encoded by the nucleic acid construct is a protein kinase
or a part thereof which changes cellular localisation upon activation. In one embodiment
the biologically active polypeptide encoded by the nucleic acid construct is a
serine/threonine protein kinase or a part thereof capable of changing intracellular
localisation upon activation. In one embodiment the biologically active polypeptide
encoded by the nucleic acid construct is a tyrosine protein kinase or a part thereof
capable of changing intracellular localisation upon activation. In one embodiment the
biologically active polypeptide encoded by the nucleic acid construct is a phospholipid-

dependent serine/threonine protein kinase or a part thereof capable of changing intracellular localisation upon activation.

In a specific embodiment the constructs listed in table 1 are used in a method for

extracting quantitative information relating to an influence on a cellular response in
mechanically intact or permeablised living cells, the method comprising recording
variation in spatially distributed fluorescence emitted from the fluorophore being present
in the cells and being capable of being redistributed in a manner which is related with the
degree of the influence, and/or being modulated by a component which is capable of
being redistributed in a manner which is related to the degree of the influence, as a

change fluorescence intensity preferably measured by an instrument designed for the measurement of changes in fluorescence intensity.

Table 1 The fusion constructs of the invention by the names used herein as well as by reference to relevant SEQ ID NOs of sequences of DNA encoding the construct and full amino acid sequences.

Fusion construct	DNA sequence SEQ ID NO:	Protein Sequence SEQ ID NO:
PKAcat - F64LS65TGFP	1	2
PKCα- F64L-S65TGFP	3	4
EGFP - Erk1	5	6
EGFP - SMAD2	7	8
SMAD2 - EGFP	9	10
EGFP - VASP	11	12
EGFP - NFχβ	13	14
$NF\chi\beta$ - EGFP	15	16
EGFP - PKCβ1	17	18

As illustrated in examples 8, 9 and 11, the redistribution of PKA, and PKC can readily be detected as a variation in fluorescence intensity, as measured e.g. in the FLIPR™ instrument.

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In one embodiment any new luminophore determined to redistribute in response to an influence in a pattern similar to the pattern observed in the microscope for PKA or PKC (see examples 1, 2, 8 and 11), that is from an aggregated form to a dispersed form or from a dispersed form to an aggregated form of the luminophore as the redistribution takes place, can be predicted to be detectable as a variation in light intensity as measured, for example in the FLIPRTM instrument.

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In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a cAMP-dependent protein kinase or a part thereof capable of changing cellular localisation upon activation. In a preferred embodiment the biologically active polypeptide encoded by the nucleic acid construct is a PKAc-F64L-S65T-GFP fusion. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a cGMP-dependent protein kinase or a part thereof capable of changing cellular localisation upon activation.

- 10 In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a calmodulin-dependent serine/threonine protein kinase or a part thereof capable of changing cellular localisation upon activation.
- In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a mitogen-activated serine/threonine protein kinase or a part thereof capable of changing cellular localisation upon activation. In preferred embodiments the biologically active polypeptide encoded by the nucleic acid constructs are an ERK1-F64L-S65T-GFP fusion or an EGFP-ERK1 fusion.
- 20 In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a cyclin-dependent serine/threonine protein kinase or a part thereof capable of changing cellular localisation upon activation.
- In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein phosphatase or a part thereof capable of changing cellular localisation upon activation.

In one preferred embodiment of the invention the nucleic acid constructs may be DNA constructs.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct. In one embodiment the gene encoding GFP in the nucleic acid construct is derived from Aequorea victoria. In a preferred embodiment the gene encoding GFP in the nucleic acid construct is EGFP or a GFP variant selected from F64L-GFP, F64L-Y66H-GFP and F64L-S65T-GFP.

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In preferred embodiments of the invention the DNA constructs which can be identified by any of the DNA sequences shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15 and 17 or are variants of these sequences capable of encoding the same fusion polypeptide or a fusion polypeptide which is biologically equivalent thereto, e.g. an isoform, or a splice variant or a homologue from another species.

The present invention describes a method that may be used to establish a screening program for the identification of biologically active substances that directly or indirectly affects intracellular signalling pathways and because of this property are potentially useful as medicaments. Based on measurements in living cells of the redistribution of spatially resolved luminescence from luminophores which undergo a change in distribution upon activation or deactivation of an intracellular signalling pathway the result of the individual measurement of each substance being screened indicates its potential biological activity.

In one embodiment of the invention the screening program is used for the identification of a biologically toxic substance as defined herein that exerts its toxic effect by interfering with an intracellular signalling pathway. Based on measurements in living cells of the 20 redistribution of spatially resolved luminescence from luminophores which undergo a change in distribution upon activation or deactivation of an intracellular signalling pathway the result of the individual measurement of each substance being screened indicates its potential biologically toxic activity. In one embodiment of a screening program a compound that modulates a component of an intracellular pathway as defined 25 herein, can be found and the therapeutic amount of the compound estimated by a method according to the method of the invention. In a preferred embodiment the present invention leads to the discovery of a new way of treating a condition or disease related to the intracellular function of a biologically active polypeptide comprising administration to a patient suffering from said condition or disease of an effective amount of a compound 30 which has been discovered by any method according to the invention. In another preferred embodiment of the invention a method is established for identification of a new drug target or several new drug targets among the group of biologically active polypeptides which are components of intracellular signalling pathways.

In another embodiment of the invention an individual treatment regimen is established for the selective treatment of a selected patient suffering from an ailment where the available medicaments used for treatment of the ailment are tested on a relevant primary cell or cells obtained from said patient from one or several tissues, using a method comprising transfecting the cell or cells with at least one DNA sequence encoding a fluorescent probe according to the invention, transferring the transfected cell or cells back the said patient, or culturing the cell or cells under conditions permitting the expression of said probes and exposing it to an array of the available medicaments, then comparing changes in fluorescence patterns or redistribution patterns of the fluorescent probes in the intact living cells to detect the cellular response to the specific medicaments (obtaining a cellular action profile), then selecting one or more medicament or medicaments based on the desired activity and acceptable level of side effects and administering an effective amount of these medicaments to the selected patient.

15 The present invention describes a method that may be used to establish a screening program for back-tracking signal transduction pathways as defined herein. In one embodiment the screening program is used to establish more precisely at which level one or several compounds affect a specific signal transduction pathway by successively or in parallel testing the influence of the compound or compounds on the redistribution of spatially resolved luminescence from several of the luminophores which undergo a change in distribution upon activation or deactivation of the intracellular signalling pathway under study.

In general, a probe, i.e. a "GeneX"-GFP fusion or a GFP-"GeneX" fusion, is constructed
using PCR with "GeneX"-specific primers followed by a cloning step to fuse "GeneX" in
frame with GFP. The fusion may contain a short vector derived sequence between
"GeneX" and GFP (e.g. part of a multiple cloning site region in the plasmid) resulting in a
peptide linker between "GeneX" and GFP in the resulting fusion protein.

30 Some of the steps involved in the development of a probe include the following:

Identify the sequence of the gene. This is most readily done by searching a depository of genetic information, e.g. the GenBank Sequence Database, which is widely available and routinely used by molecular biologists. In the specific examples below the GenBank Accession number of the gene in question is provided.

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Design the gene-specific primers. Inspection of the sequence of the gene allows design of gene-specific primers to be used in a PCR reaction. Typically, the top-strand primer encompasses the ATG start codon of the gene and the following ca. 20 nucleotides, while the bottom-strand primer encompasses the stop codon and the ca. 20 preceding 5 nucleotides, if the gene is to be fused behind GFP, i.e. a GFP-"GeneX" fusion. If the gene is to be fused in front of GFP. i.e. a "GeneX"-GFP fusion, a stop codon must be avoided. Optionally, the full-length sequence of GeneX may not be used in the fusion, but merely the part that localizes and redistributes like GeneX in response to a signal. In addition to gene-specific sequences, the primers contain at least one recognition 10 sequence for a restriction enzyme, to allow subsequent cloning of the PCR product. The sites are chosen so that they are unique in the PCR product and compatible with sites in the cloning vector. Furthermore, it may be necessary to include an exact number of nucleotides between the restriction enzyme site and the gene-specific sequence in order to establish the correct reading frame of the fusion gene and/or a translation initiation 15 consensus sequence. Lastly, the primers always contain a few nucleotides in front of the restriction enzyme site to allow efficient digestion with the enzyme.

Identify a source of the gene to be amplified. In order for a PCR reaction to produce a product with gene-specific primers, the gene-sequence must initially be present in the reaction, e.g. in the form of cDNA. Information in GenBank or the scientific literature will usually indicate in which tissue(s) the gene is expressed, and cDNA libraries from a great variety of tissues or cell types from various species are commercially available, e.g. from Clontech (Palo Alto), Stratagene (La Jolla) and Invitrogen (San Diego). Many genes are also available in cloned form from The American Type Tissue Collection (Virginia).

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Optimise the PCR reaction. Several factors are known to influence the efficiency and specificity of a PCR reaction, including the annealing temperature of the primers, the concentration of ions, notably Mg²⁺ and K⁺, present in the reaction, as well as pH of the reaction. If the result of a PCR reaction is deemed unsatisfactory, it might be because the parameters mentioned above are not optimal. Various annealing temperatures should be tested, e.g. in a PCR machine with a built-in temperature gradient, available from e.g. Stratagene (La Jolla), and/or various buffer compositions should be tried, e.g. the OptiPrime buffer system from Stratagene (La Jolla).

Clone the PCR product. The vector into which the amplified gene product will be cloned and fused with GFP will already have been taken into consideration when the primers

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were designed. When choosing a vector, one should at least consider in which cell types the probe subsequently will be expressed, so that the promoter controlling expression of the probe is compatible with the cells. Most expression vectors also contain one or more selective markers, e.g. conferring resistance to a drug, which is a useful feature when one wants to make stable transfectants. The selective marker should also be compatible with the cells to be used.

The actual cloning of the PCR product should present no difficulty as it typically will be a one-step cloning of a fragment digested with two different restriction enzymes into a vector digested with the same two enzymes. If the cloning proves to be problematic, it may be because the restriction enzymes did not work well with the PCR fragment. In this case one could add longer extensions to the end of the primers to overcome a possible difficulty of digestion close to a fragment end, or one could introduce an intermediate cloning step not based on restriction enzyme digestion. Several companies offer systems for this approach, e.g. Invitrogen (San Diego) and Clontech (Palo Alto).

Once the gene has been cloned and, in the process, fused with the GFP gene, the resulting product, usually a plasmid, should be carefully checked to make sure it is as expected. The most exact test would be to obtain the nucleotide sequence of the fusion-20 gene.

Once a DNA construct for a probe has been generated, its functionality and usefulness may be evaluated by transfecting it into cells capable of expressing the probe. The fluorescence of the cell is inspected soon after, typically the next day. At this point, two features of cellular fluorescence are noted: the intensity and the sub-cellular localisation.

The intensity should usually be at least as strong as that of unfused GFP in the cells. If it is not, the sequence or quality of the probe-DNA might be faulty, and should be carefully checked.

The sub-cellular localisation is an indication of whether the probe is likely to perform well. If it localises as expected for the gene in question, e.g. is excluded from the nucleus, it can immediately go on to a functional test. If the probe is not localised soon after the transfection procedure, it may be because of overexpression at this point in time, as the

35 cell typically will have taken up very many copies of the plasmid, and localisation will

occur in time, e.g. within a few weeks, as plasmid copy number and expression level decreases. If localisation does not occur after prolonged time, it may be because the fusion to GFP has destroyed a localisation function, e.g. masked a protein sequence essential for interaction with its normal cellular anchor-protein. In this case the opposite fusion might work, e.g. if GeneX-GFP does not work, GFP-GeneX might, as two different parts of GeneX will be affected by the proximity to GFP. If this does not work, the proximity of GFP at either end might be a problem, and it could be attempted to increase the distance by incorporating a longer linker between GeneX and GFP in the DNA construct.

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If there is no prior knowledge of localisation, and no localisation is observed, it may be because the probe should not be localised at this point, because such is the nature of the protein fused to GFP. It should then be subjected to a functional test.

In a functional test, the cells expressing the probe are treated with at least one compound known to perturb, usually by activating, the signalling pathway on which the probe is expected to report by redistributing itself within the cell. If the redistribution is as expected, e.g. if prior knowledge tell that it should translocate from location X to location Y, it has passed the first critical test. In this case it can go on to further characterisation and quantification of the response.

If it does not perform as expected, it may be because the cell lacks at least one component of the signalling pathway, e.g. a cell surface receptor, or there is species incompatibility, e.g. if the probe is modelled on sequence information of a human gene product, and the cell is of hamster origin. In both instances one should identify other cell types for the testing process where these potential problems would not apply.

If there is no prior knowledge about the pattern of redistribution, the analysis of the redistribution will have to be done in greater depth to identify what the essential and indicative features are, and when this is clear, it can go on to further characterisation and quantification of the response. If no feature of redistribution can be identified, the problem might be as mentioned above, and the probe should be retested under more optimal cellular conditions. If the probe does not perform under optimal cellular conditions, then it's back to the drawing board.

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The process of developing an image-based redistribution assay begins with either the unplanned experimental observation that a redistribution phenomenon can be visualised, or the design of a probe specifically to follow a redistribution phenomenon already known to occur. In either event, the first and best exploratory technique is for a trained scientist or technician to observe the phenomenon. Even with the rapid advances in computing technology, the human eye-brain combination is still the most powerful pattern recognition system known, and requires no advance knowledge of the system in order to detect potentially interesting and useful patterns in raw data. This is especially if those data are presented in the form of images, which are the natural "data type" for human visual processing. Because human visual processing operates most effectively in a relatively narrow frequency range. i.e., we cannot see either very fast or very slow changes in our visual field, it may be necessary to record the data and play it back with either time dilation or time compression.

15 Some luminescence phenomena cannot be seen directly by the human eye. Examples include polarisation and fluorescence lifetime. However, with suitable filters or detectors, these signals can be recorded as images or sequences of images and displayed to the human in the fashion just described. In this way, patterns can be detected and the same methods can be applied.

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Once the redistribution has been determined to be a reproducible phenomenon, one or more data sets are generated for the purpose of developing a procedure for extracting the quantitative information from the data. In parallel, the biological and optical conditions are determined which will give the best quality raw data for the assay. This can become an iterative process: it may be necessary to develop a quantitative procedure in order to assess the effect on the assay of manipulating the assay conditions.

The data sets are examined by a person or persons with knowledge of the biological phenomenon and skill in the application of image processing techniques. The goal of this exercise is to determine or at least propose a method that will reduce the image or sequence of images constituting the record of a "response" to a value corresponding to the degree of the response. Using either interactive image processing software or an image processing toolbox and a programming language, the method is encoded as a procedure or algorithm that takes the image or images as input and generates the

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degree of response (in any units) as its output. Some of the criteria for evaluating the validity of a particular procedure are:

Does the degree of the response vary in a biologically significant fashion, i.e., does it show the known or putative dependence on the concentration of the stimulating agent or condition?

Is the degree of response reproducible, i.e., does the same concentration or level of stimulating agent or condition give the same response with an acceptable variance? Is the dynamic range of the response sufficient for the purpose of the assay? If not, can a change in the procedure or one of its parameters improve the dynamic range? Does the procedure exhibit any clear "pathologies", i.e., does it give ridiculous values for the response if there are commonly occurring imperfections in the imaging process? Can these pathologies be eliminated, controlled, or accounted for? Can the procedure deal with the normal variation in the number and/or size of cells in an image?

In some cases the method may be obvious; in others, a number of possible procedures may suggest themselves. Even if one method appears clearly superior to others, optimisation of parameters may be required. The various procedures are applied to the data set and the criteria suggested above are determined, or the single procedure is applied repeatedly with adjustment of the parameter or parameters until the most satisfactory combination of signal, noise, range, etc. are arrived at. This is equivalent to the calibration of any type of single-channel sensor.

The number of ways of extracting a single value from an image are extremely large, and thus an intelligent approach must be taken to the initial step of reducing this number to a small, finite number of possible procedures. This is not to say that the procedure arrived at is necessarily the best procedure - but a global search for the best procedure is simply out of the question due to the sheer number of possibilities involved.

Image-based assays are no different than other assay techniques in that their usefulness is characterised by parameters such as the specificity for the desired component of the sample, the dynamic range, the variance, the sensitivity, the concentration range over which the assay will work, and other such parameters. While it is not necessary to

characterise each and every one of these before using the assay, they represent the only way to compare one assay with another.

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The final step is then to see whether there exists a possibility to increase the throughput of the assay to improve its utility as the basis of a screening program. In order to do this, a dose-response relationship is established based on quantification of the responses caused by a particular influence, representative of the underlying intracellular signalling process, using the methods described above and in the examples. The dose-response relationship for the particular influence is then compared to the dose-response relationship obtained by performing the same assay in an instrument which allows parallel monitoring of all wells in a microtiter plate such as a FLIPR™ or an ordinary imaging or fluorescence plate reader for microtiter plates. If a good correlation between the dose-response relationships obtained from the two different measurement systems is obtained, it can be said that the parallel measurement mode has been validated (see examples 8, 9 and 11). This implies that it can be used as the primary basis for a screening program with the potential benefit of screening a significantly higher number of substances for their influence on the response per unit of time.

In the present specification and claims, the term "an influence" covers any influence to
which the cellular response comprises a redistribution. Thus, e.g., heating, cooling, pH,
high pressure, low pressure, humidifying, or drying are influences on the cellular
response on which the resulting redistribution can be quantified, but as mentioned
above, perhaps the most important influences are the influences of contacting or
incubating the cells with substances which are known or suspected to exert an influence
on the cellular response involving a redistribution contribution. In another embodiment of
the invention the influence could be substances from a compound drug library.

In the present context, the term "green fluorescent protein" is intended to indicate a protein which, when expressed by a cell, emits fluorescence upon exposure to light of the correct excitation wavelength (cf. [(Chalfie, M. et al. (1994) Science 263, 802-805)]). In the following, GFP in which one or more amino acids have been substituted, inserted or deleted is most often termed "modified GFP". "GFP" as used herein includes wild-type GFP derived from the jelly fish *Aequorea victoria* and modifications of GFP, such as the blue fluorescent variant of GFP disclosed by Heim et al. (1994). Proc.Natl.Acad.Sci. 91:26, pp 12501-12504, and other modifications that change the spectral properties of the GFP

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fluorescence, or modifications that exhibit increased fluorescence when expressed in cells at a temperature above about 30°C described in PCT/DK96/00051, published as WO 97/11094 on 27 March 1997 and hereby incorporated by reference, and which comprises a fluorescent protein derived from *Aequorea* Green Fluorescent Protein (GFP) or any functional analogue thereof, wherein the amino acid in position 1 upstream from the chromophore has been mutated to provide an increase of fluorescence intensity when the fluorescent protein of the invention is expressed in cells. Preferred GFP variants are F64L-GFP, F64L-Y66H-GFP and F64L-S65T-GFP. An especially preferred variant of GFP for use in all the aspects of this invention is EGFP (DNA encoding EGFP which is a F64L-10 S65T variant with codons optimized for expression in mammalian cells is available from Clontech, Palo Alto, plasmids containing the EGFP DNA sequence, cf. GenBank Acc. Nos. U55762, U55763).

The term "intracellular signalling pathway" and "signal transduction pathway" are

intended to indicate the co-ordinated intracellular processes whereby a living cell
transduce an external or internal signal into cellular responses. Said signal transduction
will involve an enzymatic reaction said enzymes include but are not limited to protein
kinases, GTPases, ATPases, protein phosphatases, phospholipases and cyclic
nucleotide phosphodiesterases. The cellular responses include but are not limited to
gene transcription, secretion, proliferation, mechanical activity, metabolic activity, cell
death.

The term "second messenger" is used to indicate a low molecular weight component involved in the early events of intracellular signal transduction pathways.

The term "luminophore" is used to indicate a chemical substance that has the property of emitting light either inherently or upon stimulation with chemical or physical means. This includes but is not limited to fluorescence, bioluminescence, phosphorescence, and chemiluminescence.

The term "mechanically intact living cell" is used to indicate a cell which is considered living according to standard criteria for that particular type of cell such as maintenance of normal membrane potential, energy metabolism, proliferative capability, and has not experienced any physically invasive treatment designed to introduce external substances into the cell such as microinjection.

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In the present context, the term "permeabilised living cell" is used to indicate cells where a pore forming agent such as Streptolysin O or Staphylococcus Aureus α -toxin has been applied and thereby incorporated into the plasma membrane in the cells. This creates 5 proteinaceous pores with a defined pore size in the plasma membranes of the exposed cells. Pores could also be made by electroporation, i.e. exposing the cells to high voltage discharges, a procedure that creates small holes in the plasma membrane by coagulating integral membrane proteins. Treatment with a mild detergent such as saponin may accomplish the same thing. Common to all these treatments are that pores 10 are formed only in the plasma membrane without affecting the integrity of cytoplasmic structural elements and organelles. The term living in this context means that the permeabilised cells bathed in a solution mimicking the intracellular milieu still have functional organelles, such as actively respiring mitochondria and endoplasmic reticulum that can take up and release calcium ions, and functional structural elements. The benefit of this method is that substances that normally can not traverse the plasma membrane, but most likely exert their influence intracellularly, can be introduced and their influence studied without cumbersome microinjection of the substances into single cells. Using this method the response to an influence can be recorded from many cells simultaneously.

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In the present context, the term "permeabilisation" is intended to indicate the selective disruption of the plasma membrane barrier so that soluble substances freely mobile in the cytosol are lost from the cells. The permeabilisation can be achieved as described above under "permeabilised living cells" or by using other chemical detergents such as 25 Triton X-100 or digitonin in carefully titrated amounts.

The term "physiologically relevant", when applied to an experimentally determined redistribution of an intracellular component, as measured by a change in the luminescence properties or distribution, is used to indicate that said redistribution can be explained in terms of the underlying biological phenomenon which gives rise to the redistribution.

The terms "image processing" and "image analysis" are used to describe a large family of digital data analysis techniques or combination of such techniques which reduce ordered arrays of numbers (images) to quantitative information describing those ordered

arrays of numbers. When said ordered arrays of numbers represent measured values from a physical process, the quantitative information derived is therefore a measure of the physical process.

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The term "fluorescent probe" is used to indicate a fluorescent fusion polypeptide comprising a GFP or any functional part thereof which is N- or C-terminally fused to a biologically active polypeptide as defined herein, optionally via a peptide linker consisting of one or more amino acid residues, where the size of the linker peptide in itself is not critical as long as the desired functionality of the fluorescent probe is maintained. A fluorescent probe according to the invention is expressed in a cell and basically mimics the physiological behaviour of the biologically active polypeptide moiety of the fusion

the physiological behaviour of the biologically active polypeptide moiety of the fusion polypeptide.

The term "mammalian cell" is intended to indicate any living cell of mammalian origin. 15 The cell may be an established cell line, many of which are available from The American Type Culture Collection (ATCC, Virginia, USA) or a primary cell with a limited life span derived from a mammalian tissue. including tissues derived from a transgenic animal, or a newly established immortal cell line derived from a mammalian tissue including transgenic tissues, or a hybrid cell or cell line derived by fusing different cell types of 20 mammalian origin e.g. hybridoma cell lines. The cells may optionally express one or more non-native gene products, e.g. receptors, enzymes, enzyme substrates, prior to or in addition to the fluorescent probe. Preferred cell lines include but are not limited to those of fibroblast origin, e.g. BHK. CHO, BALB, or of endothelial origin, e.g. HUVEC, BAE (bovine artery endothelial). CPAE (cow pulmonary artery endothelial), HLMVEC 25 (human lung microvascular endothelial cells) or of pancreatic origin, e.g. RIN, INS-1, MIN6, bTC3, aTC6, bTC6, HIT, or of hematopoietic origin, e.g.primary isolated human monocytes, macrophages, neutrophils, basophils, eosinophils and lyphocyte populations, AML-193, HL-60, RBL-1, adipocyte origin, e.g. 3T3-L1, neuronal/neuroendocrine origin. e.g. AtT20, PC12, GH3, muscle origin, e.g. SKMC, A10, 30 C2C12, renal origin, e.g. HEK 293. LLC-PK1.

The term "hybrid polypeptide" is intended to indicate a polypeptide which is a fusion of at least a portion of each of two proteins, in this case at least a portion of the green fluorescent protein, and at least a portion of a catalytic and/or regulatory domain of a protein kinase. Furthermore a hybrid polypeptide is intended to indicate a fusion

polypeptide comprising a GFP or at least a portion of the green fluorescent protein that contains a functional fluorophore, and at least a portion of a biologically active polypeptide. Thus, GFP may be N- or C-terminally tagged to a biologically active polypeptide, optionally via a linker portion or linker peptide consisting of a sequence of one or more amino acids. The hybrid polypeptide or fusion polypeptide may act as a fluorescent probe in intact living cells carrying a DNA sequence encoding the hybrid polypeptide under conditions permitting expression of said hybrid polypeptide.

The term "kinase" is intended to indicate an enzyme that is capable of phosphorylating a cellular component.

The term "protein kinase" is intended to indicate an enzyme that is capable of phosphorylating serine and/or threonine and/or tyrosine in peptides and/or proteins.

15 The term "phosphatase" is intended to indicate an enzyme that is capable of dephosphorylating phosphoserine and/or phosphothreonine and/or phosphotyrosine in peptides and/or proteins.

The term "cyclic nucleotide phosphodiesterase" is intended to indicate an enzyme that is capable of inactivating the second messengers cAMP and cGMP by hydrolysis of their 3'-ester bond.

In the present context, the term "biologically active polypeptide" is intended to indicate a polypeptide affecting intracellular processes upon activation, such as an enzyme which is active in intracellular processes or a portion thereof comprising a desired amino acid sequence which has a biological function or exerts a biological effect in a cellular system. In the polypeptide one or several amino acids may have been deleted, inserted or replaced to alter its biological function, e.g. by rendering a catalytic site inactive. Preferably, the biologically active polypeptide is selected from the group consisting of proteins taking part in an intracellular signalling pathway, such as enzymes involved in the intracellular phosphorylation and dephosphorylation processes including kinases, protein kinases and phosphorylases as defined herein, but also proteins making up the cytoskeleton play important roles in intracellular signal transduction and are therefore included in the meaning of "biologically active polypeptide" herein. More preferably, the biologically active polypeptide is a protein which according to its state as activated or

non-activated changes localisation within the cell, preferably as an intermediary component in a signal transduction pathway. Included in this preferred group of biologically active polypeptides are cAMP dependent protein kinase A.

5 The term "a substance having biological activity" is intended to indicate any sample that has a biological function or exerts a biological effect in a cellular system. The sample may be a sample of a biological material such as a sample of a body fluid including blood, plasma, saliva, milk, urine, or a microbial or plant extract, an environmental sample containing pollutants including heavy metals or toxins. or it may be a sample containing a compound or mixture of compounds prepared by organic synthesis or genetic techniques.

The phrase "any change in fluorescence" means any change in absorption properties, such as wavelength and intensity, or any change in spectral properties of the emitted light, such as a change of wavelength, fluorescence lifetime, intensity or polarisation, or any change in the intracellular localisation of the fluorophore. It may thus be localised to a specific cellular component (e.g. organelle, membrane, cytoskeleton, molecular structure) or it may be evenly distributed throughout the cell or parts of the cell.

- The term "organism" as used herein indicates any unicellular or multicellular organism preferably originating from the animal kingdom including protozoans, but also organisms that are members of the plant kingdoms, such as algae, fungi. bryophytes, and vascular plants are included in this definition.
- 25 The term "nucleic acid" is intended to indicate any type of poly- or oligonucleic acid sequence, such as a DNA sequence, a cDNA sequence, or an RNA sequence.

The term "biologically equivalent" as it relates to proteins is intended to mean that a first protein is equivalent to a second protein if the cellular functions of the two proteins may substitute for each other, e.g. if the two proteins are closely related isoforms encoded by different genes, if they are splicing variants, or allelic variants derived from the same gene, if they perform identical cellular functions in different cell types, or in different species. The term "biologically equivalent" as it relates to DNA is intended to mean that a first DNA sequence encoding a polypeptide is equivalent to a second DNA sequence

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encoding a polypeptide if the functional proteins encoded by the two genes are biologically equivalent.

The term "higher throughput" is intended to mean an increased number of experiments 5 per time unit per person performing the actual experiments.

The term "high throughput screening assay" as used herein is intended to mean the process of performing a screening assay with at least 100 individual experiments where compounds are tested for their influence on the redistribution of a luminophore in one working day for one person skilled in the art. In a preferred embodiment the high throughput screening assay involves at least 500 individual experiments such as 750, 1000, 2000, 5000, or even 10.000 individual experiments in one working day for a person skilled in the art.

- The phrase "back-tracking of a signal transduction pathway" is intended to indicate a process for defining more precisely at what level a signal transduction pathway is affected, either by the influence of chemical compounds or a disease state in an organism. Consider a specific signal transduction pathway represented by the bioactive polypeptides A B C D, with signal transduction from A towards D. When investigating all components of this signal transduction pathway compounds or disease states that influence the activity or redistribution of only D can be considered to act on C or downstream of C whereas compounds or disease states that influence the activity or redistribution of C and D, but not of A and B can be considered to act downstream of B.
 - 25 The term "fixed cells" is used to mean cells treated with a cytological fixative such as glutaraldehyde or formaldehyde, treatments that serve to chemically cross-link and stabilise soluble and insoluble proteins within the structure of the cell. Once in this state, such proteins cannot be lost from the structure of the now-dead cell.
 - 30 In the present context a "screening assay" is intended to mean any measurement protocol, including materials, cells. instruments, chemicals, reagents, detection units, calibration and quantification procedures used to measure a response from mechanically intact or permeabilised living cells relevant to influences on an intracellular pathway.

The term "dose-response relationship" and "screening programme" is in the present context intended to mean a clear correlation between the quantified response of cells in a screening assay to application of an influence, such as a compound, and the concentration of the applied influence. The response to the influence may be both an upregulation and a down-regulation of the quantified parameter used in the screening assay.

In the present context, the term "physiology" is intended to mean the normal function of biological and biochemical processes inside cells, between cells and in the whole organism or animal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. CHO cells expressing the PKAc-F64L-S65T-GFP hybrid protein have been treated in HAM's F12 medium with 50 μM forskolin at 37°C. The images of the GFP fluorescence in these cells have been taken at different time intervals after treatment, which were: a) 40 seconds b) 60 seconds c) 70 seconds d) 80 seconds. The fluorescence changes from a punctate to a more even distribution within the (non-nuclear) cytoplasm.

- 20 Figure 2. Time-lapse analysis of forskolin induced PKAc-F64L-S65T-GFP redistribution. CHO cells, expressing the PKAc-F64L-S65T-GFP fusion protein were analysed by time-lapse fluorescence microscopy. Fluorescence micrographs were acquired at regular intervals from 2 min before to 8 min after the addition of agonist. The cells were challenged with 1 μM forskolin immediately after the upper left image was acquired (t=0).
- 25 Frames were collected at the following times: i) 0, ii) 1, iii) 2, iv) 3, v) 4 and vi) 5 minutes. Scale bar 10 μ m.
- Figure 3. Time-lapse analyses of PKAc-F64L-S65T-GFP redistribution in response to various agonists. The effects of 1 μM forskolin (A), 50 μM forskolin (B), 1mM dbcAMP (C) and 100 μM IBMX (D) (additions indicated by open arrows) on the localisation of the PKAc-F64L-S65T-GFP fusion protein were analysed by time-lapse fluorescence microscopy of CHO/PKAc-F64L-S65T-GFP cells. The effect of addition of 10 μM forskolin (open arrow), followed shortly by repeated washing with buffer (solid arrow), on the localisation of the PKAc-F64L-S65T-GFP fusion protein was analysed in the same

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cells (E). In a parallel experiment, the effect of adding 10 μM forskolin and 100 μM IBMX (open arrow) followed by repeated washing with buffer containing 100 μM IBMX (solid arrow) was analysed (F). Removing forskolin caused PKAc-F64L-S65T-GFP fusion protein to return to the cytoplasmic aggregates while this is prevented by the continued presence of IBMX (F). The effect of 100 nM glucagon (Fig 3G, open arrow) on the localisation of the PKAc-F64L-S65T-GFP fusion protein is also shown for BHK/GR, PKAc-F64L-S65T-GFP cells. The effect of 10 μM norepinephrine (H), solid arrow, on the localisation of the PKAc-F64L-S65T-GFP fusion protein was analysed similarly, in transiently transfected CHO, PKAc-F64L-S65T-GFP cells, pretreated with 10 μM forskolin, open arrow, to increase [cAMP]. N.B. in Fig 3H the x-axis counts the image numbers, with 12 seconds between images. The raw data of each experiment consisted of 60 fluorescence micrographs acquired at regular intervals including several images acquired before the addition of buffer or agonist. The charts (A-G) each show a quantification of the response seen through all the 60 images. performed as described in analysis method 2. The change in total area of the highly fluorescent aggregates, relative

Figure 4. Dose-response curve (two experiments) for forskolin-induced redistribution of the PKAc-F64L-S65T-GFP fusion.

to the initial area of fluorescent aggregates is plotted as the ordinate in all graphs in

Figure 3, versus time for each experiment. Scale bar 10 μm.

Figure 5. Time from initiation of a response to half maximal (t_{1/2max}) and maximal (t_{max}) PKAc-F64L-S65T-GFP redistribution. The data was extracted from curves such as that shown in "Figure 2." All t_{1/2max} and t_{max} values are given as mean±SD and are based on a total of 26-30 cells from 2-3 independent experiments for each forskolin concentration. Since the observed redistribution is sustained over time, the t_{max} values were taken as the earliest time point at which complete redistribution is reached. Note that the values do not relate to the degree of redistribution.

Figure 6. Parallel dose-response analyses of forskolin induced cAMP elevation and PKAc-F64L-S65T-GFP redistribution. The effects of buffer or 5 increasing concentrations of forskolin on the localisation of the PKAc-F64L-S65T-GFP fusion protein in CHO/PKAc-F64L-S65T-GFP cells, grown in a 96 well plate, were analysed as described above. Computing the ratio of the SD's of fluorescence micrographs taken of the same field of cells, prior to and 30 min after the addition of forskolin, gave a reproducible measure of

PKAc-F64L-S65T-GFP redistribution. The graph shows the individual 48 measurements and a trace of their mean±s.e.m at each forskolin concentration. For comparison, the effects of buffer or 8 increasing concentrations of forskolin on [cAMP]_i was analysed by a scintillation proximity assay of cells grown under the same conditions. The graph shows a trace of the mean ± s.e.m of 4 experiments expressed in arbitrary units.

Figure 7. BHK cells stably transfected with the human muscarinic (hM1) receptor and the PKCα-F64L-S65T-GFP fusion. Carbachol (100 μM added at 1.0 second) induced a transient redistribution of PKCα-F64L-S65T-GFP from the cytoplasm to the plasma membrane. Images were taken at the following times: a) 1 second before carbachol addition, b) 8.8 seconds after addition and c) 52.8 seconds after addition.

Figure 8. BHK cells stably transfected with the hM1 receptor and PKCα-F64L-S65T-GFP fusion were treated with carbachol (1 μM, 10 μM, 100 μM). In single cells intracellular [Ca²+] was monitored simultaneously with the redistribution of PKCα-F64L-S65T-GFP. Dashed line indicates the addition times of carbachol. The top panel shows changes in the intracellular Ca²+ concentration of individual cells with time for each treatment. The middle panel shows changes in the average cytoplasmic GFP fluorescence for individual cells against time for each treatment. The bottom panel shows changes in the fluorescence of the periphery of single cells, within regions that specifically include the circumferential edge of a cell as seen in normal projection, the best regions for monitoring changes in the fluorescence intensity of the plasma membrane.

Figure 9. The hERK1-F64L-S65T-GFP fusion expressed in HEK293 cells treated with 100 μM of the MEK1 inhibitor PD98059 in HAM F-12 (without serum) for 30 minutes at 37 °C. The nuclei empty of fluorescence during this treatment. The same cells as in (a) following treatment with 10 % foetal calf serum for 15 minutes at 37 °C.

Time profiles for the redistribution of GFP fluorescence in HEK293 cells following treatment with various concentrations of EGF in Hepes buffer (HAM F-12 replaced with Hepes buffer directly before the experiment). Redistribution of fluorescence is expressed as the change in the ratio value between areas in nucleus and cytoplasm of single cells. Each time profile is the mean for the changes seen in six single cells.

Bar chart for the end-point measurements, 600 seconds after start of EGF treatments, of fluorescence change (nucleus:cytoplasm) following various concentrations of EGF.

Figure 10. The SMAD2-EGFP fusion expressed in HEK293 cells starved of serum overnight in HAM F-12. HAM F-12 was then replaced with Hepes buffer pH 7.2 immediately before the experiment. Scale bar is 10 μ m.

HEK 293 cells expressing the SMAD2-EGFP fusion were treated with various concentration of TGF-beta as indicated, and the redistribution of fluorescence monitored against time. The time profile plots represent increases in fluorescence within the nucleus, normalised to starting values in each cell measured. Each trace is the time profile for a single cell nucleus.

A bar chart representing the end-point change in fluorescence within nuclei (after 850 seconds of treatment) for different concentrations of TGF-beta. Each bar is the value for a single nucleus in each treatment.

Figure 11. The VASP-F64L-S65T-GFP fusion in CHO cells stably transfected with the human insulin receptor. The cells were starved for two hours in HAM F-12 without serum, then treated with 10% foetal calf serum. The image shows the resulting redistribution of fluorescence after 15 minutes of treatment. GFP fluorescence becomes localised in structures identified as focal adhesions along the length of actin stress fibres.

Figure 12. Dose-response relationship for the translocation of PKC α -GFP in BHKhM1 cells stimulated with the muscarininc agonist carbamylcholine using a FLIPRTM to do the actual experiments.

Figure 13. Dose-response relationship for the translocation of PKAc-GFP in CHO/PKAc-F64L-S65T-GFP cells stimulated with forskolin using a FLIPR™ to do the actual experiments.

Figure 14. CHO cells stably expressing the human insulin receptor and mouse cPKA labeled with S65T-GFP were more thoroughly investigated in the FLIPR™ instrument. A forskolin (a substance that increases Adenylate cyclase production of cAMP in the cells) dose-response was created where six separate wells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC (area under the curve) for 9 min of stimulation.

Conclusion: Redistribution of mouse cPKA - BioST can be detected in the FLIPR[™] despite the fact that the whole wells, containing around 50-100 000 cells, are illuminated and imaged simultaneously with a spatial resolution that is far from capable of resolving

single cells or subcellular events. The method can be used as a real time measurement of cAMP levels in the cells and as a screening assay to measure effects of ligands to G-protein coupled receptors linked to Gi and Gq type G-proteins.

- 5 Figure 15. Dose-response relationship for the disappearance of fluorescence from permeabilised CHO/PKAc-F64L-S65T-GFP when previously exposed to different doses of forskolin.
- Figure 16. CHO cells stably expressing the human insulin receptor and human PKC beta
 10 1 labeled with EGFP were investigated in the microscope. A dose-response was created
 where a set of cells were imaged over time for each concentration. The changes in
 fluorescence were calculated as AUC for 4 min of stimulation. From the images the
 following data were extracted:
- Whole image: Just analysing the change in intensity in the whole images taking both cells and background.
 - Single cell: 5 separate cells were analysed after background compensation. The analysis was made on the entire cell.
 - Cytoplasm: The same 5 cells as above were analysed after background compensation. the analysis was made on a small region in the cytoplasm close to the nucleus.
- 20 Conclusion: Redistribution of human PKC beta 1 EGFP can only be detected if a subregion of each cell is analysed. The event is clearly visible when the image series is viewed as a movie but if the whole image change in fluorescence or the change in fluorescence in entire cells are analysed the redistribution cannot be detected.
- Figure 17. CHO cells stably expressing the human insulin receptor and human PKC beta 1 labeled with EGFP were investigated in the FLIPR™. A dose-response was created where six separate wells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 5 min of stimulation.
- Conclusion: Redistribution of human PKC beta 1 EGFP can be detected in the FLIPRTM despite the fact that the whole wells, containing around 50-100 000 cells, are illuminated and imaged by a detector that has a resolution far below that needed to resolve single cells or subcellular structures. This phenomenon can clearly not be predicted from the microscope data in Figure 16.

Figure 18 CHO cells stably expressing the insulin receptor and a human NFkB – GFP protein hybrid were stimulated with different concentrations of IL-1 for 1 h, then washed with a hypoosmotic buffer (TRIS-base 10mM, MgCl2 2mM, PMSF(Phenyl methyl sulfonyl fluoride) 0.5mM, pH 7.4) and placed on the microscope. A sequence of images were acquired during the addition of 0.05% Triton X-100 and subsequent gentle mixing after a short incubation period. The treatment causes the cell membranes to rupture leaving the fraction of NFkB-GFP that has translocated to the nucleus behind whereas the cytoplasmic amount of the probe leaves the cells more quickly and immediately becomes infinitely diluted in the surrounding medium (out of focus - this part of the total fluorescence from the probe is thereby lost). At a defined time point before and after this treatment a total intensity value for the whole image was extracted. To normalize each

- treatment a total intensity value for the whole image was extracted. To normalize each experiment, the after value was divided by the before value meaning that a higher ratio was found in cells where more NFkB had translocated to the nucleus and thereby contributed to the total fluorescence after permeabilisation.
- 15 <u>Conclusion:</u> the present protocol is a good example of the possibility of revealing translocation of a fluorescent probe from the cytosol to the nucleus or translocation from the nucleus to the cytosol.

EXAMPLES

EXAMPLE 1 Construction, testing and implementation of an assay for cAMP based on PKA activation.

5 Useful for monitoring the activity of signalling pathways that lead to altered concentrations of cAMP, e.g. activation of G-protein coupled receptors which couple to G-proteins of the G_S or G_I class.

The catalytic subunit of the murine cAMP dependent protein kinase (PKAc) was fused C-terminally to a F64L-S65T derivative of GFP. The resulting fusion (PKAc-F64L-S65T-

10 GFP) was used for monitoring *in vivo* the translocation and thereby the activation of PKA.

To construct the PKAc-F64L-S65T-GFP fusion, convenient restriction endonuclease sites were introduced into the cDNAs encoding murine PKAc (Gen Bank Accession number: M12303) and F64L-S65T-GFP (sequence disclosed in WO 97/11094) by

15 polymerase chain reaction (PCR). The PCR reactions were performed according to standard protocols with the following primers:

5'PKAc:

TTggACACAAgCTTTggACACCCTCAggATATgggCAACgCCgCCgCCGCCAAg, 3'PKAc:

20 gTCATCTTCTCgAgTCTTTCAggCgCgCCCAAACTCAgTAAACTCCTTgCCACAC 5'GFP:

TTggACACAAgCTTTggACACggCgCgCCCATgAgTAAAggAgAACTTTTC 3'GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT.

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The PKAc amplification product was then digested with HindIII+AscI and the F64L-S65T-GFP product with AscI+XhoI. The two digested PCR products were subsequently ligated with a HindIII+XhoI digested plasmid (pZeoSV® mammalian expression vector, Invitrogen, San Diego, CA, USA). The resulting fusion construct (SEQ ID NO:1 and 2)

30 was under control of the SV40 promoter.

Transfection and cell culture conditions:

Chinese hamster ovary cells (CHO), were transfected with the plasmid containing the PKAc-F64L-S65T-GFP fusion using the calcium phosphate precipitate method in HEPES-buffered saline (Sambrook *et al.*, 1989). Stable transfectants were selected using 1000 µg Zeocin/ml (Invitrogen) in the growth medium (DMEM with 1000 mg

- 5 glucose/l, 10 % fetal bovine serum (FBS), 100 μg penicillin-streptomycin mixture ml⁻¹, 2 mM L-glutamine purchased from Life Technologies Inc., Gaithersburg, MD, USA). Untransfected CHO cells were used as the control. To assess the effect of glucagon on fusion protein translocation, the PKAc-F64L-S65T-GFP fusion was stably expressed in baby hamster kidney cells overexpressing the human glucagon receptor (BHK/GR cells).
- 10 Untransfected BHK/GR cells were used as the control. Expression of GR was maintained with 500 μg G418/ml (Neo marker) andPKAc-F64L-S65T-GFP was maintained with 500 μg Zeocin/ml (Sh ble marker). CHO cells were also simultaneously co-transfected with vectors containing the PKAc-F64L-S65T-GFP fusion and the human α2a adrenoceptor (hARa2a).
- 15 For fluorescence microscopy, cells were allowed to adhere to Lab-Tek chambered coverglasses (Nalge Nunc Int., Naperville, IL, USA) for at least 24 hours and cultured to about 80% confluence. Prior to experiments, the cells were cultured over night without selection pressure in HAM F-12 medium with glutamax (Life Technologies), 100 μg penicillin-streptomycin mixture ml⁻¹ and 0.3 % FBS. This medium has low autofluorescence enabling fluorescence microscopy of cells straight from the incubator.
 - Monitoring activity of PKA activity in real time:
 - Image aquisition of live cells were gathered using a Zeiss Axiovert 135M fluorescence microscope fitted with a Fluar 40X. NA: 1.3 oil immersion objective and coupled to a
- 25 Photometrics CH250 charged coupled device (CCD) camera. The cells were illuminated with a 100 W HBO arc lamp. In the light path was a 470±20 nm excitation filter, a 510 nm dichroic mirror and a 515±15 nm emission filter for minimal image background. The cells were maintained at 37°C with a custom built stage heater.
 - Images were processed and analysed in the following manner:
- 30 Method 1: Stepwise procedure for quantitation of translocation of PKA:

 The image was corrected for dark current by performing a pixel-by-pixel subtraction of a dark image (an image taken under the same conditions as the actual image, except the camera shutter is not allowed to open).

The image was corrected for non-uniformity of the illumination by performing a pixel-by-pixel ratio with a flat field correction image (an image taken under the same conditions as the actual image of a uniformly fluorescent specimen).

The image histogram, i.e., the frequency of occurrence of each intensity value in the 5 image, was calculated.

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A smoothed, second derivative of the histogram was calculated and the second zero is determined. This zero corresponds to the inflection point of the histogram on the high side of the main peak representing the bulk of the image pixel values.

The value determined in step 4 was subtracted from the image. All negative values were discarded.

The variance (square of the standard deviation) of the remaining pixel values was determined. This value represents the "response" for that image.

Scintillation proximity assay (SPA) for independent quantitation of cAMP.

15 Method 2: Alternative method for quantitation of PKA redistribution:

The fluorescent aggregates are segmented from each image using an automatically found threshold based on the maximisation of the information measure between the object and background. The *a priori* entropy of the image histogram is used as the information measure.

20 The area of each image occupied by the aggregates is calculated by counting pixels in the segmented areas.

The value obtained in step 2 for each image in a series, or treatment pair, is normalised to the value found for the first (unstimulated) image collected. A value of zero (0) indicates no redistribution of fluorescence from the starting condition. A value of one (1)

25 by this method equals full redistribution.

manufacturer.

Cells were cultured in HAM F-12 medium as described above, but in 96-well plates. The medium was exchanged with Ca²⁺-HEPES buffer including 100 µM IBMX and the cells were stimulated with different concentrations of forskolin for 10 min. Reactions were stopped with addition of NaOH to 0.14 M and the amount of cAMP produced was measured with the cAMP-SPA kit, RPA538 (Amersham) as described by the

Manipulating intracellular levels of cAMP to test the PKAc-F64L-S65T-GFP fusion.

(data not shown).

WO 00/23615 PCT/DK99/00562

The following compounds were used to vary cAMP levels: Forskolin, an activator of adenylate cyclase; dbcAMP, a membrane permeable cAMP analog which is not degraded by phosphodiesterase; IBMX, an inhibitor of phosphodiesterase. CHO cells stably expressing the PKAc-F64L-S65T-GFP, showed a dramatic

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- 5 translocation of the fusion protein from a punctate distribution to an even distribution throughout the cytoplasm following stimulation with 1 μM forskolin (n=3), 10 μM forskolin (n=4) and 50 μM forskolin (n=4) (Fig 1), or dbcAMP at 1mM (n=6).
- Fig. 2 shows the progression of response in time following treatment with 1 μM forskolin. Fig. 3 gives a comparison of the average temporal profiles of fusion protein redistribution and a measure of the extent of each response to the three forskolin concentrations (Fig. 3A, E, B), and to 1 mM dbcAMP (fig 3C) which caused a similar but slower response, and to addition of 100 μM IBMX (n=4, Fig. 3D) which also caused a slow response, even in the absence of adenylate cyclase stimulation. Addition of buffer (n=2) had no effect
- As a control for the behaviour of the fusion protein, F64L-S65T-GFP alone was expressed in CHO cells and these were also given 50 μM forskolin (n=5); the uniform diffuse distribution characteristic of GFP in these cells was unaffected by such treatment (data not shown).

The forskolin-induced translocation of PKAc-F64L-S65T-GFP showed a dose-response relationship (Fig 4 and 6), see quantitative procedures above.

Reversibility of PKAc-F64L-S65T-GFP translocation.

The release of the PKAc probe from its cytoplasmic anchoring hotspots was reversible. Washing the cells repeatedly (5-8 times) with buffer after 10µM forskolin treatment

- completely restored the punctate pattern within 2-5 min (n=2, Fig. 3E). In fact the fusion protein returned to a pattern of fluorescent cytoplasmic aggregates virtually indistinguishable from that observed before forskolin stimulation.
 - To test whether the return of fusion protein to the cytoplasmic aggregates reflected a decreased [cAMP]_i, cells were treated with a combination of 10 μ M forskolin and 100 μ M
- 30 IBMX (n=2) then washed repeatedly (5-8 times) with buffer containing 100 μM IBMX (Fig. 3F). In these experiments, the fusion protein did not return to its prestimulatory localisation after removal of forskolin.

Testing the PKA-F64L-S65T-GFP probe with physiologically relevant agents.

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To test the probe's response to receptor activation of adenylate cyclase, BHK cells stably transfected with the glucagon receptor and the PKA-F64L-S65T-GFP probe were exposed to glucagon stimulation. The glucagon receptor is coupled to a G_S protein which activates adenylate cyclase, thereby increasing the cAMP level. In these cells, addition of 100 nM glucagon (n=2) caused the release of the PKA-F64L-S65T-GFP probe from the cytoplasmic aggregates and a resulting translocation of the fusion protein to a more even cytoplasmic distribution within 2-3 min (Fig. 3G). Similar but less pronounced effects were seen at lower glucagon concentrations (n=2, data not shown). Addition of buffer (n=2) had no effect over time (data not shown).

10 Transiently transfected CHO cells expressing hARα2a and the PKA-F64L-S65T-GFP probe were treated with 10 μM forskolin for 7.5 minutes, then. in the continued presence of forskolin, exposed to 10 μM norepinephrine to stimulate the exogenous adrenoreceptors, which couple to a G_I protein, which inhibit adenylate cyclase. This treatment led to reappearance of fluorescence in the cytoplasmic aggregates indicative of a decrease in [cAMP]_I (Fig. 3H).

Fusion protein translocation correlated with [cAMP],

As described above, the time it took for a response to come to completion was dependent on the forskolin dose (Fig. 5) In addition the degree of responses was also dose-dependent. To test the PKA-F64L-S65T-GFP fusion protein translocation in a semi high through-put system, CHO cells stably transfected with the PKA-F64L-S65T-GFP fusion was stimulated with buffer and 5 increasing doses of forskolin (n=8). Using the image analysis algorithm described above (Method 1), a dose-response relationship was observed in the range from 0.01-50 μM forskolin (Fig. 6). A half-maximal stimulation was observed at about 2 μM forskolin. In parallel, cells were stimulated with buffer and 8 increasing concentrations of forskolin (n=4) in the range 0.01-50 μM. The amount of cAMP produced was measured in an SPA assay. A steep increase was observed between 1 and 5 μM forskolin coincident with the steepest part of the curve for fusion protein translocation (also Fig. 6).

30

EXAMPLE 2 Probe for detection of PKC activity

Construction of PKC-GFP fusion:

The probe was constructed by ligating two restriction enzyme treated polymerase chain reaction (PCR) amplification products of the cDNA for murine PKCα (GenBank

Accession number: M25811) and F64L-S65T-GFP (sequence disclosed in WO 97/11094) respectively. Taq® polymerase and the following oligonucleotide primers were used for PCR;

5'mPKCα:

5 TTggACACAAgCTTTggACACCCTCAggATATggCTgACgTTTACCCggCCAACg 3'mPKCα:

gTCATCTTCTCgAgTCTTTCAggCgCgCCCTACTgCACTTTgCAAgATTgggTgC, 5'F64L-S65T-GFP:

TTggACACAAgCTTTggACACggCgCGCCATgAgTAAAggAgAAGATTTTC,

10 3'F64L-S65T-GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT.

The hybrid DNA strand was inserted into the pZeoSV® mammalian expression vector as a HindIII-XhoI casette as described in example 1.

- BHK cells expressing the human M1 receptor under the control of the inducible metallothionine promoter and maintained with the dihydrofolate reductase marker were transfected with the PKCα-F64L-S65T-GFP probe using the calcium phosphate precipitate method in HEPES buffered saline (HBS [pH 7.10]). Stable transfectants were selected using 1000 μg Zeocin®/ml in the growth medium (DMEM with 1000 mg
- glucose/l, 10 % foetal bovine serum (FBS), 100 μg penicillin-streptomycin mixture ml-1, 2 mM l-glutamine). The hM1 receptor and PKCα-F64L-S65T-GFP fusion protein were maintained with 500 nM methotrexate and 500 μg Zeocin®/ml respectively. 24 hours prior to any experiment, the cells were transferred to HAM F-12 medium with glutamax, 100 μg penicillin-streptomycin mixture ml⁻¹ and 0.3 % FBS. This medium relieves
- 25 selection pressure, gives a low induction of signal transduction pathways and has a low autofluorescence at the relevant wavelength enabling fluorescence microscopy of cells straight from the incubator.

Method 1: Monitoring the PKC α activity in real time:

- Digital images of live cells were gathered using a Zeiss Axiovert 135M fluorescence microscope fitted with a 40X, NA: 1.3 oil immersion objective and coupled to a Photometrics CH250 charged coupled device (CCD) camera. The cells were illuminated with a 100 W arc lamp. In the light path was a 470±20 nm excitation filter, a 510 nm dichroic mirror and a 515±15 nm emission filter for minimal image background. The cells
- 35 were kept and monitored to be at 37°C with a custom built stage heater.

Images were analyzed using the IPLab software package for Macintosh.

Upon stimulation of the M1-BHK cells, stably expressing the PKCα-F64L-S65T-GFP fusion, with carbachol we observed a dose-dependent transient translocation from the cytoplasm to the plasma membrane (Fig. 7a,b,c). Simultaneous measurement of the 5 cytosolic free calcium concentration shows that the carbachol-induced calcium

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Stepwise procedure for quantification of translocation of PKCa:

mobilisation precedes the translocation (Fig. 8).

The image was corrected for dark current by performing a pixel-by-pixel subtraction of a dark image (an image taken under the same conditions as the actual image, except the 10 camera shutter is not allowed to open).

The image was corrected for non-uniformity of the illumination by performing a pixel-bypixel ratio with a flat field correction image (an image taken under the same conditions as the actual image of a uniformly fluorescent specimen).

A copy of the image was made in which the edges are identified. The edges in the image are found by a standard edge-detection procedure - convolving the image with a kernel which removes any large-scale unchanging components (i.e., background) and accentuates any small-scale changes (i.e., sharp edges). This image was then converted to a binary image by threshholding. Objects in the binary image which are too small to represent the edges of cells were discarded. A dilation of the binary image was 20 performed to close any gaps in the image edges. Any edge objects in the image which were in contact with the borders of the image are discarded. This binary image represents the edge mask.

Another copy of image was made via the procedure in step 3. This copy was further processed to detect objects which enclose "holes" and setting all pixels inside the holes 25 to the binary value of the edge, i.e., one. This image represents the whole cell mask.

The original image was masked with the edge mask from step 3 and the sum total of all pixel values is determined.

The original image was masked with the whole cell mask from step 4 and the sum total of all pixel values was determined.

30 The value from step 5 was divided by the value from step 6 to give the final result, the fraction of fluorescence intensity in the cells which was localized in the edges.

EXAMPLE 3 Probes for detection of mitogen activated protein kinase Erk1 redistribution.

Useful for monitoring signalling pathways involving MAPK, e.g. to identify compounds which modulate the activity of the pathway in living cells.

- 5 Erk1, a serine/threonine protein kinase, is a component of a signalling pathway that is activated by e.g. many growth factors.
 - Probes for detection of ERK-1 activity in real time within living cells:
 - The extracellular signal regulated kinase (ERK-1, a mitogen activated protein kinase, MAPK) is fused N- or C-terminally to a derivative of GFP. The resulting fusions
- 10 expressed in different mammalian cells are used for monitoring *in vivo* the nuclear translocation, and thereby the activation, of ERK1 in response to stimuli that activate the MAPK pathway.
 - The human Erk1 gene (GenBank Accession number: X60188) was amplified using PCR according to standard protocols with primers
- 15 Erk1-top
 - 5'-TAGAATTCAACCATGGCGGCGGCGGCGGCG-3' and Erk1-bottom/+stop
 - 5'-TAGGATCCCTAGGGGGCCTCCAGCACTCC-3'.
- The PCR product was digested with restriction enzymes EcoR1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with EcoR1 and BamH1. This produces an EGFP-Erk1 fusion (SEQ ID NOs: 5 and 6) under the control of a CMV promoter.
 - The plamid containing the EGFP-Erk1 fusion was transfected into HEK293 cells employing the FUGENE transfection reagent (Boehringer Mannheim). Prior to
- experiments the cells were grown to 80%-90% confluency 8 well chambers in DMEM with 10% FCS. The cells were washed in plain HAM F-12 medium (without FCS), and then incubated for 30-60 minutes in plain HAM F-12 (without FCS) with 100 micromolar PD98059, an inhibitor of MEK1, a kinase which activates Erk1; this step effectively empties the nucleus of EGFP-Erk1. Just before starting the experiment, the HAM F-12
- 30 was replaced with Hepes buffer following a wash with Hepes buffer. This removes the PD98059 inhibitor; if blocking of MEK1 is still wanted (e.g. in control experiments), the inhibitor is included in the Hepes buffer.
 - The experimental setup of the microscope was as described in example 1.
 - 60 images were collected with 10 seconds between each, and with the test compound
- 35 added after image number 10.

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Addition of EGF (1-100 nM) caused within minutes a redistribution of EGFP-Erk1 from the cytoplasm into the nucleus (Fig. 9a,b).

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The response was quantitated as described below and a dose-dependent relationship between EGF concentration and nuclear translocation of EGFP-Erk1 was found (Fig.

9c,d). Redistribution of GFP fluorescence is expressed in this example as the change in the ratio value between areas in nuclear versus cytoplasmic compartments of the cell. Each time profile is the average of nuclear to cytoplasmic ratios from six cells in each treatment.

10 EXAMPLE 4 Probes for detection of Smad2 redistribution.

Useful for monitoring signalling pathways activated by some members of the transforming growth factor-beta family, e.g. to identify compounds which modulate the activity of the pathway in living cells.

Smad 2, a signal transducer, is a component of a signalling pathway that is induced by some members of the TGFbeta family of cytokines.

- a) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers Smad2-top
- 5'-GTGAATTCGACCATGTCGTCCATCTTGCCATTC-3'
- 20 and Smad2-bottom/+stop
 - 5'-GTGGTACCTTATGACATGCTTGAGCAACGCAC-3'.

The PCR product was digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-C1 (Clontech; Palo Alto; GenBank Accession number U55763) digested with EcoR1 and Acc65I. This produces an EGFP-Smad2 fusion (SEQ ID NOs: 7 and 8) under

- 25 the control of a CMV promoter.
 - b) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers Smad2-top
 - 5'-GTGAATTCGACCATGTCGTCCATCTTGCCATTC-3'
- 30 and Smad2-bottom/-stop
 - 5'-GTGGTACCCATGACATGCTTGAGCAACGCAC-3'.

The PCR product was digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-N1 (Clontech, Palo Alto; GenBank Accession number U55762) digested with EcoR1 and Acc65I. This produces a Smad2-EGFP fusion (SEQ ID NOs:9 and 10) under

35 the control of a CMV promoter.

The plasmid containing the EGFP-Smad2 fusion was transfected into HEK293 cells, where it showed a cytoplasmic distribution. Prior to experiments the cells were grown in 8 well Nunc chambers in DMEM with 10% FCS to 80% confluence and starved overnight in HAM F-12 medium without FCS.

5 For experiments, the HAM F-12 medium was replaced with Hepes buffer pH 7.2. The experimental setup of the microscope was as described in example 1. 90 images were collected with 10 seconds between each, and with the test compound added after image number 5.

After serum starvation of cells, each nucleus contains less GFP fluorescence than the surrounding cytoplasm (Fig. 10a). Addition of TGFbeta caused within minutes a redistribution of EGFP-Smad2 from the cytoplasma into the nucleus (Fig. 10b). The redistribution of fluorescence within the treated cells was quantified simply as the fractional increase in nuclear fluorescence normalised to the starting value of GFP fluorescence in the nucleus of each unstimulated cell and displayed a dose dependent change in response to TGFβ (fig. 10c).

EXAMPLE 5 Probes for detection of VASP redistribution.

Useful for monitoring signalling pathways involving rearrangement of cytoskeletal elements, e.g. to identify compounds which modulate the activity of the pathway in living cells. VASP, a phosphoprotein, is a component of cytoskeletal structures, which redistributes in response to signals that affect focal adhesions.

The human VASP gene (GenBank Accession number: Z46389) was amplified using PCR according to standard protocols with primers VASP-top

5'-GGGAAGCTTCCATGAGCGAGACGGTCATC-3'

25 and VASP-bottom/+stop

5'-CCCGGATCCTCAGGGAGAACCCCGCTTC-3'.

The PCR product was digested with restriction enzymes Hind3 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Hind3and BamH1. This produces an EGFP-VASP fusion (SEQ ID NOs:11 and 12) under

30 the control of a CMV promoter.

The resulting plasmid was transfected into CHO cells expressing the human insulin receptor using the calcium-phosphate transfection method. Prior to experiments, cells were grown in 8 well Nunc chambers and starved overnight in medium without FCS. Experiments are performed in a microscope setup as described in example 1.10% FCS was added to the cells and images were collected. The EGFP-VASP fusion was

redistributed from a somewhat even distribution near the periphery into more localised structures, identified as focal adhesion points (Fig. 11).

EXAMPLE 7 Probes for detection of NFkappaB redistribution.

- 5 Useful for monitoring signalling pathways leading to activation of NFkappaB, e.g. to identify compounds which modulate the activity of the pathway in living cells. NFkappaB, an activator of transcription, is a component of signalling pathways that are responsive to a varity of inducers including cytokines, lymphokines, and some immunosuppressive agents.
- 10 a) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers NFkappaB-top
 - 5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC-3' and NFkappaB-bottom/+stop
- 5'-GTGGATCCTTAGGAGCTGATCTGACTCAGCAG-3'.
 The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Xho1 and BamH1. This produces an EGFP-NFkappaB fusion (SEQ ID NOs:13 and 14)

under the control of a CMV promoter.

- 20 b) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers NFkappaB-top
 - 5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC-3' and NFkappaB-bottom/-stop
- 25 5'-GTGGATCCAAGGAGCTGATCTGACTCAGCAG-3'.
 - The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-N1 (Clontech, Palo Alto; GenBank Accession number U55762) digested with Xho1 and BamH1. This produces an NFkappaB-EGFP fusion (SEQ ID NOs: 15 and 16) under the control of a CMV promoter.
- The resulting plasmids are transfected into a suitable cell line, e.g. Jurkat, in which the EGFP-NFkappaB probe and/or the NFkappaB-EGFP probe should change its cellular distribution from cytoplasmic to nuclear in response to activation of the signalling pathway with e.g. IL-1.

CHO cells stably expressing the insulin receptor and a human NFkB – GFP protein hybrid were stimulated with different concentrations of IL-1 for 1 hour, then washed with a hypoosmotic buffer (TRIS-base 10mM, MgCl2 2mM, PMSF(Phenyl methyl sulfonyl fluoride) 0.5mM, pH 7.4) and placed on the microscope. A sequence of images were acquired during the addition of 0.05% Triton X-100 and subsequent gentle mixing after a short incubation period. The treatment causes the cell membranes to rupture leaving the fraction of NFkB-GFP that has translocated to the nucleus behind whereas the cytoplasmic amount of the probe leaves the cells more quickly and immediately becomes infinitely diluted in the surrounding medium (out of focus - this part of the total fluorescence from the probe is thereby lost). At a defined time point before and after this treatment a total intensity value for the whole image was extracted. To normalize each experiment, the after value was divided by the before value, meaning that a higher ratio was found in cells where more NFkB had translocated to the nucleus and thereby contributed to the total fluorescence after permeabilisation, the actual data from such an experiment run in duplicate is shown in Figure 18.

Conclusion: the present protocol is a good example of the possibility of revealing translocation of a fluorescent probe from the cytosol to the nucleus or translocation from the nucleus to the cytosol by using a measurement immediately before and after plasma membrane permeabilisation recorded a s an image sequence.

EXAMPLE 8 real-time redistribution of protein kinase C lpha

Measurement of the real-time redistribution of protein kinase C α isoform-GFP fusion (PKC α -GFP, SEQ ID NOs: 3 and 4) in response to carbamylcholine stimulation of the muscarinic M1 receptor; 96 parallel redistribution measurements in microtiter plates.

BHK cells were stably expressing a recombinant human muscarinic type 1 receptor, under the selection with 500 μg/ml Methotrexate, and also a PKCα-GFP construct (KaA 048), under the selection of 500 nM Zeocin. The cells were grown in 96-well plates (Packard ViewPlate, black with transparent bottom), washed and preincubated in a Hank's Buffered Salt solution (HBSS) without phenol red, with 20 mM HEPES and 5.5
 mM glucose.

The plate was measured in a FLIPR™ (Fluorescence Imaging Plate Reader) from Molecular Devices. The 488 nm emission line from an argon ion laser, run at between 0.4 and 0.8 W output, was used to excite fluorescence form the GFP. Emission wavelengths were collected through a 510 to 565 nm band pass filter.

The cells were challenged with three doses of carbamylcholine, an M1 receptor agonist known from previous studies to give a microscopically detectable redistribution of the PKCα-GFP construct [(Almholt *et al.* 1997)]. Measurements were made every 10 seconds for 5 minutes. After data handling including normalisation of baseline fluorescence for the different wells. background subtraction and averaging the 6 wells used for each concentration the data presented in figure 14 were obtained. It can clearly be seen (Fig 12) that carbamylcholine gave a time- and dose-dependent, and transient, decrease in fluorescence very similar to the time- and dose-dependent profile seen in microscopic fluorescence measurements [(see Almholt *et al.* 1997)]. This experiment was repeated twice on the same batch of cells with similar results.

EXAMPLE 9 real-time redistribution of cyclic-AMP dependent protein kinase catalytic subuit-GFP fusion

Measurement of the real-time redistribution of cyclic-AMP dependent protein kinase catalytic subuit-GFP fusion (C-GFP^{LT} SEQ ID NOs: 1 and 2) in response to forskolin stimulation of the adenylate cyclase: 96 parallel redistribution measurements in microtiter plates.

CHO cells were stably transfected with hybrid DNA for the PKA catalytic subunit-F64L+S65T GFP (C-GFP^{LT}) fusion protein, and were typically under continuous selection with 1000 µg/ml zeocin (Invitrogen). The cells were grown without selection for 2 days in 96-well plates (Packard ViewPlate. black with transparent bottom), washed and preincubated in a Hank's Buffered Salt solution (HBSS) without phenol red, with 20 mM HEPES and 5.5 mM glucose.

The plate was measured in a FLIPR™ (Fluorescence Imaging Plate Reader) from

Molecular Devices. The 488 nm emission line from an argon ion laser, run at between
0.4 and 0.8 W output, was used to excite fluorescence from the GFP. Emission
wavelengths were collected through a 510 to 565 nm band pass filter.

The cells were challenged with three doses of forskolin (Fig 13), an adenylate cyclase agonist known from previous studies to give a microscopically detectable redistribution of the C-GFP^{LT} construct. Measurements were made every 10 seconds for over 6 minutes from the point of addition of forskolin. After data handling including normalisation of baseline fluorescence for the different wells, background subtraction and averaging the 6 wells used for each concentration the data presented below were obtained. It can clearly be seen in figure 15 that forskolin gave a time- and dose-dependent decrease in fluorescence very similar to the time- and dose-dependent profile seen in microscopic

fluorescence measurements. This experiment was repeated twice on the same batch of cells with similar results. As can be seen in figure 14, a more extensive dose-response test gives at hand that this method is both sensitive and reproducible enough to use as the basis for a high throughput screening assay.

5 EXAMPLE 10 cyclic-AMP dependent protein kinase catalytic subuit-GFP fusion

Measurement of the redistribution response of cyclic-AMP dependent protein kinase catalytic subuit-GFP fusion (C-GFP^{LT} SEQ ID NOs: 1 and 2) after forskolin stimulation of the adenylate cyclase; measurement of the change in total fluorescence upon permeabilisation of agonist-treated cells.

CHO cells were stably transfected with hybrid DNA for the PKA catalytic subunit-F64L+S65T GFP (C-GFP^{LT}) fusion protein, and were typically under continuous selection with 1000 μ g/ml zeocin (Invitrogen). For the experiments reported here, cells were grown without selection to 90% confluence in 8-well tissue culture-treated Lab-Tek®

chambered coverglass units (chambers, obtained from Nunc, Inc. Illinois, USA). Immediately prior to the experiment growth medium was washed from the cells and replaced with 200 μ l HEPES buffer per well.

For the results reported here, chambers were measured using a cooled CCD camera (KAF1400 chip, Photometrics Ltd., USA) attached to an inverted microscope (Diaphot 300, Nikon, Japan) equipped with a x40 oil-immersion Fluar lens, NA 1.4. Cells were illuminated with 450-490 nm light from a 50 W HBO lamp, and emitted light collected between 510-560 nm.

The cells were challenged with four doses of forskolin, an adenylate cyclase agonist known from previous studies to give a microscopically detectable redistribution of the C-GFP^{LT} construct. Images were collected at 10-second intervals for a period of 10 minutes for each treatment. Six minutes after the addition of forskolin or buffer control, Triton-X100 was added to a final concentration of 0.1%. The detergent releases freely mobile C-GFP^{LT} from the cells. The change in fluorescence resulting from this loss was measured after 1 minute of equilibration. After data handling including background subtraction and normalisation to pre-detergent values, the data presented in figure 16 were obtained. Permeabilisation caused decreases in fluorescence, the magnitude of which were dependent on the forskolin treatments. This experiment was repeated twice on the same batch of cells with similar results.

EXAMPLE 11 Probes for detection of PKC β 1 redistribution.

Useful for monitoring signalling pathways involving Protein Kinase C, e.g. to identify compounds which modulate the activity of the pathway in living cells.

PKCbeta1, a serine/threonine protein kinase, is closely related to PKCalpha and

- 5 PKCbeta2 but not identical; it is a component of a signalling pathway which is activated by elevation of intracellular calcium concomitant with an increase in diacylglycerol species.
- a) The human PKCbeta1 gene (GenBank Accession number: X06318) was amplified
 using PCR according to standard protocols with primers
 PKCβ1-top

GTCTCGAGGCAAGATGGCTGACCC

and PKCβ1-bottom

GTGGATCCCTACACATTAATGACAAACTCTGGG.

- The PCR product was digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Xho1 and BamH1. This produces an EGFP-PKCβ1 fusion (SEQ ID NOs: 17 and 18) under the control of a CMV promoter.
- b) CHO cells stably expressing the human insulin receptor and human PKC beta 1 labeled with EGFP were investigated in the microscope. A dose-response was created where a set of cells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 4 min of stimulation.
 - It can be seen in figure 16 that using microscopic measurements, redistribution of human PKC beta 1 EGFP can only be detected if a subregion of each cell is analysed. The
 - event is clearly visible when the image series is viewed as a movie but if the whole image changes in fluorescence or the changes in fluorescence in entire cells are analysed the redistribution cannot be detected.
 - CHO cells stably expressing the human insulin receptor and human PKC beta 1 labelled with EGFP were investigated in the $FLIPR^{TM}$. A dose-response was created where six
 - 30 separate wells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 5 min of stimulation. As shown in figure 17 redistribution of human PKC beta 1 − EGFP can be detected in the FLIPR™ instrument despite the fact that the whole wells, containing around 50-100 000 cells, are illuminated and imaged with a resolution far below what is needed to resolve single cells or
 - 35 subcellular compartments. This phenomenon can clearly not be predicted from the

microscope data in Figure 16. Based on these observations it is clear that a screening assay can be established in the FLIPRTM instrument. It might even be possible to establish a high throughput screening assay with further optimisation.

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VOL)



International Patent Application No. PCT/DK99/00562

Our ref: 22129PC1, Improved method for redistribution

BioImage A/S

5 CLAIMS

1. A method for extracting quantitative information relating to an influence on redistribution of at least one component in the cell in mechanically intact or permeabilised living cells, the method comprising

recording variation in spatially distributed light emitted from a luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence,

- as a change in light intensity wherein the illumination is provided by a laser which is scanned in a raster fashion over some or all of the spatial limitations being measured, the scanning taking place at a rate substantially faster than the measurement process such that the illumination appears to the measurement process to be continuous in time and spatially uniform over the region being measured.
- 2. A method according to claim 1, wherein the quantitative information which is indicative of the degree of the cellular response to the influence or the result of the influence on the subcellular component is extracted from the recorded variation according to a predetermined calibration based on responses or results, recorded in the same manner, to known degrees of a relevant specific influence.
- 3. A method according to claims 1 or 2, wherein the influence comprises contact between the mechanically intact or permeabilised living cells and a chemical substance and/or incubation of the mechanically intact or permeabilised living cells with a chemical substance.
- 4. A method according to any of claims 1-3, wherein the cells comprise a group of cells contained within a spatial limitation.
- 5. A method according to any of claims 1-4, wherein the cells comprise multiple groups of cells contained within multiple spatial limitations.

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- 6. A method according to any of claims 1-5, wherein the spatial limitations are spatial limitations arranged in one or more arrays on a common carrier.
- 7. A method according to claim 6, wherein the spatial limitations are wells in a plate of microtiter type.
- 5 8. A method according to any of claims 1-7, wherein the redistribution results in quenching of fluorescence, the quenching being measured as a decrease in the intensity of the fluorescence.
 - 9. A method according to any of claims 1-8, wherein the redistribution results in energy transfer, the energy transfer being measured as a change in the intensity of the luminescence.
 - 10. A method according to any of claims 1-8, wherein the intensity of the light being recorded is a function of the fluorescence lifetime, polarisation, wavelength shift, or other property which is modulated as a result of the underlying cellular response.
 - 11. A method according to any of claims 1-10, wherein the light to be measured passes through a filter which selects the desired component of the light to be measured and rejects other components.
 - 12. A method according to any of claims 1-11, wherein the flourescence comes from a fluorophore encoded by and expressed from a nucleotide sequence harboured in the cells.
- 13. A method according to any of the preceding claims, wherein the flourescence comes 20 from a luminescent polypeptide, such as GFP.
 - 14. A method according to any of the preceding claims, wherein the luminescent polypeptide could be a GFP selected from the group consisting of green fluorescent proteins having the F64L such as F64L-GFP, F64L-Y66H-GFP, F64L-S65T-GFP, and EGFP.
 - 15. A method according to any of claims 1-14, wherein the cells are selected from the group consisting of fungal cells, such as yeast cells; invertebrate cells including insect cells; and vertebrate cells, such as mammalian cells.
 - 16. A method according to claim 15, wherein the mechanically intact or permeabilised living cells are mammalian cells which, during the time period over which the influence is observed, are incubated at a temperature of 30°C or above, preferably at a temperature of claims after telephone interview.22129pc1.claims.1.doc

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from 32°C to 39°C, more preferably at a temperature of from 35°C to 38°C, and most preferably at a temperature of about 37°C.

- 17. A method according to any of claims 1-16, used as a screening program.
- 18. A method according claim 17, wherein the method is a screening program for the identification of a biologically active substance that directly or indirectly affects an intracellular signalling pathway and is potentially useful as a medicament, wherein the result of the individual measurement of each substance being screened which indicates its potential biological activity is based on measurement of the redistribution of spatially resolved luminescence in living cells and which undergoes a change in distribution upon activation of an intracellular signalling pathway.
 - 19. A method according to claim 17, wherein the method is a screening program for the identification of a biologically toxic substance as defined herein that exerts its toxic effect by interfering with an intracellular signalling pathway, wherein the result of the individual measurement of each substance being screened which indicates its potential biologically toxic activity is based on measurement of the redistribution of said fluorescent probe in living cells and which undergoes a change in distribution upon activation of an intracellular signalling pathway.
 - 20. A set of data relating to an influence on a cellular response in mechanically intact or permeabilised living cells, obtained by a method according to any of claims 1-19.

Figure 1



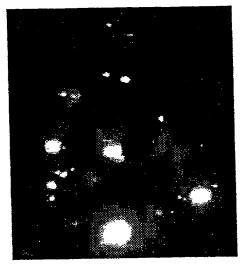


Fig. 1 a

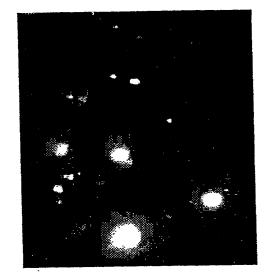


Fig. 1 b

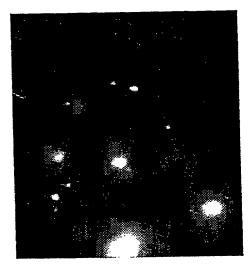


Fig. 1 c



Fig. 1 d

Fig. 1

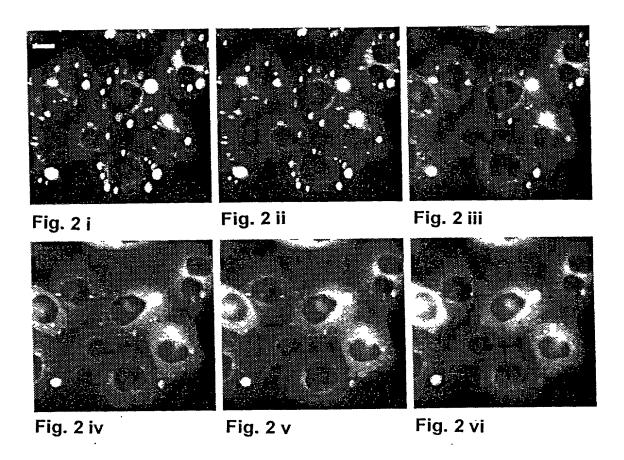


Fig. 2

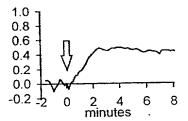


Fig. 3 A

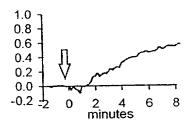


Fig. 3 C

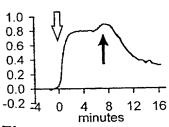


Fig. 3 E

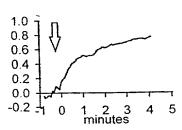


Fig. 3 G

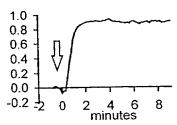


Fig. 3 B

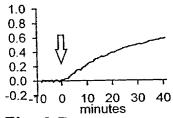


Fig. 3 D

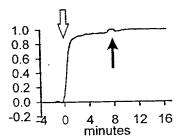


Fig. 3 F

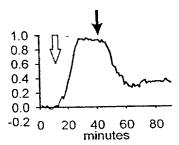


Fig. 3 H

Fig. 3
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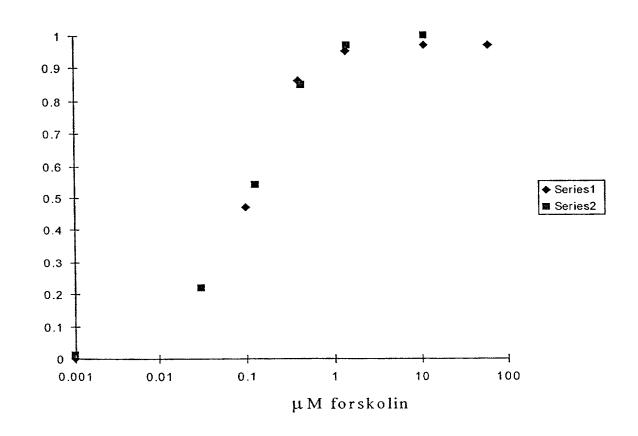


Fig. 4
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[forskolin]µM	$t_{1/2max}/s$	$t_{\rm max}/\dot{s}$
1	115±21	310±31
10	69±14	224±47
50	47±10	125±28

Fig. 5

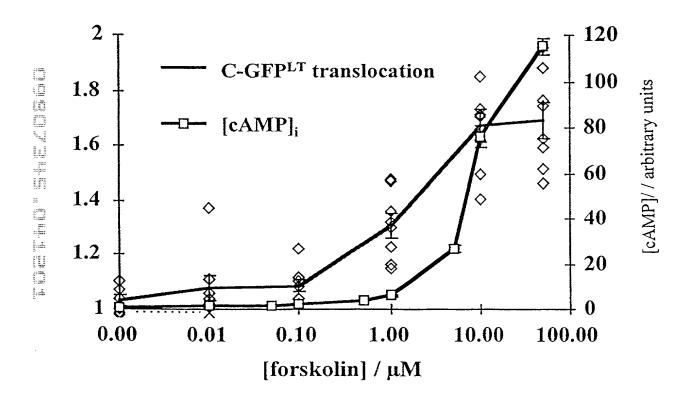


Fig. 6



Fig. 7 a

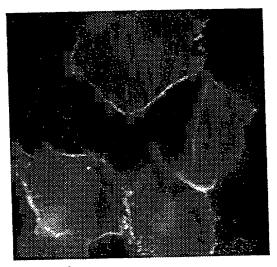


Fig. 7 b



Fig. 7 c

Fig. 7
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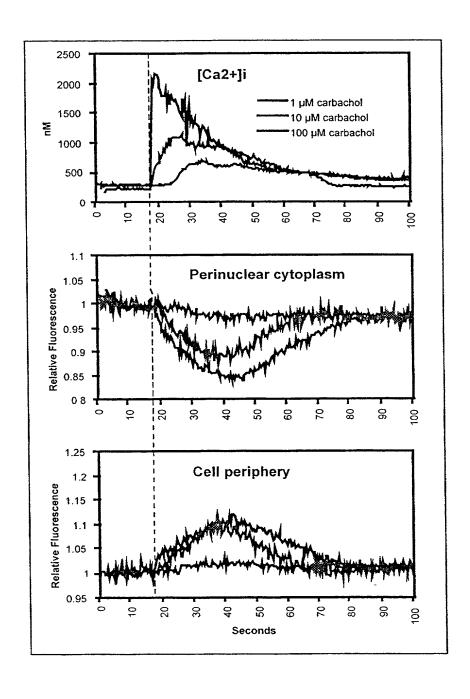
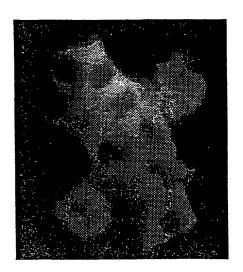


Fig. 8

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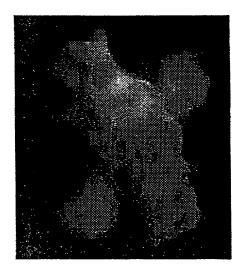


Fig. 9 a

Fig. 9 b

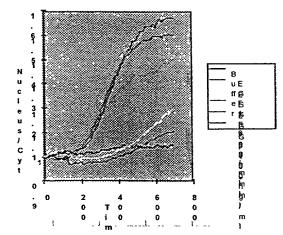


Fig. 9 c

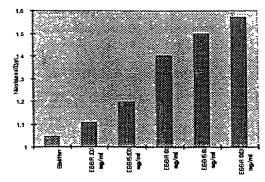


Fig. 9 d

Fig. 9
SUBSTITUTE SHEET (RULE 26)

Fig. 10 a

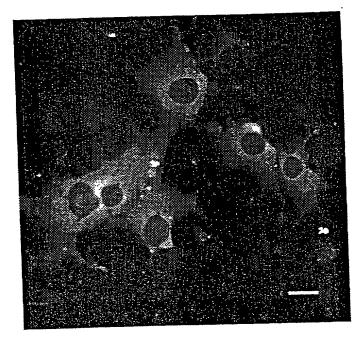


Fig. 10 b

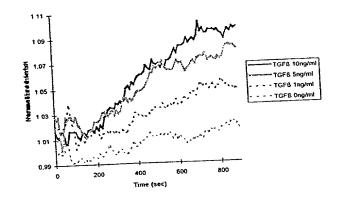


Fig. 10 c

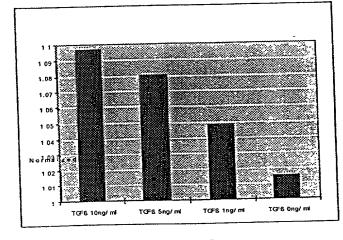


Fig. 10 SUBSTITUTE SHEET (RULE 26)

WO 00/23615

PCT/DK99/00562

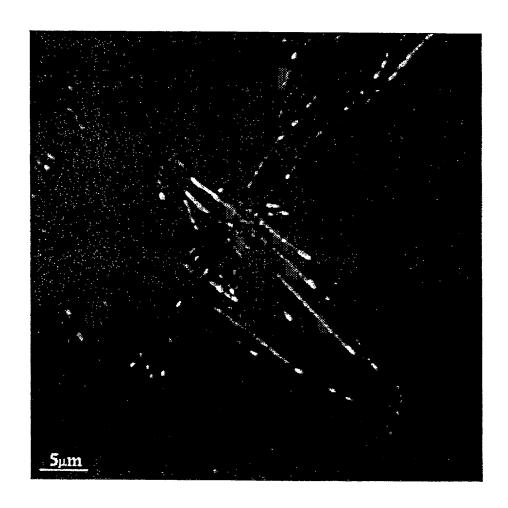


Fig. 11 SUBSTITUTE SHEET (RULE 26)

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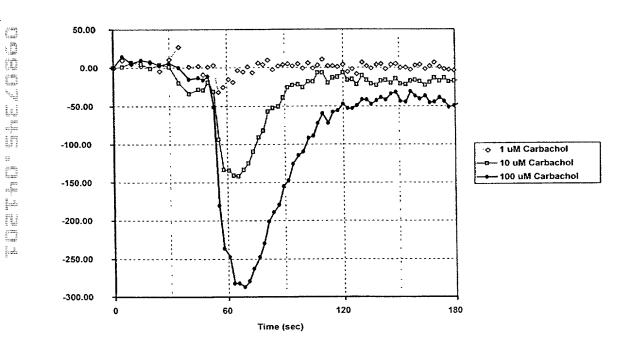


Fig. 12

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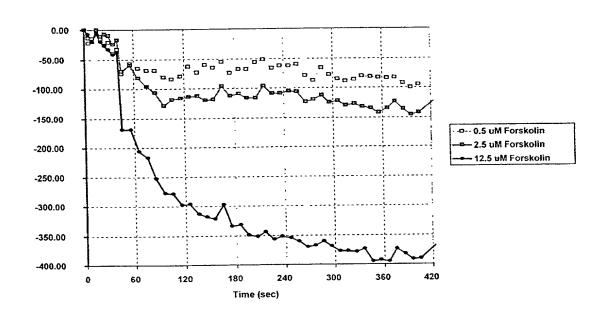


Fig. 13

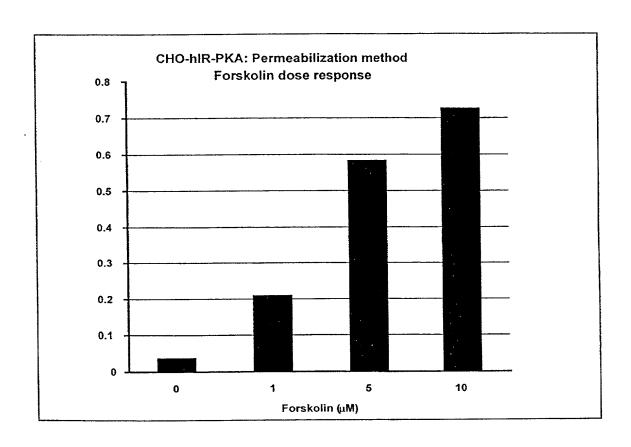


Fig. 14

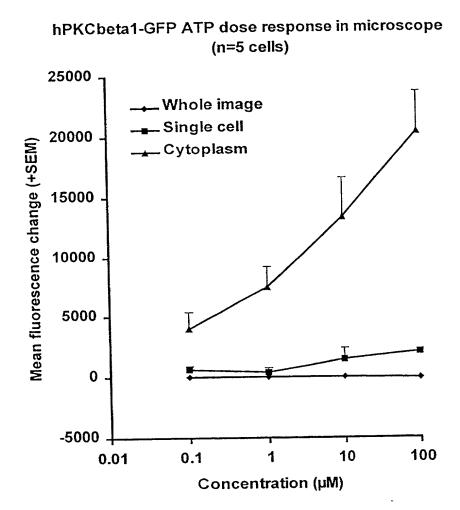


Fig. 15
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)

hPKCbeta1-GFP ATP dose-response in FLIPR (n=6)

1200

1000

Mean

Mean

0.01

Concentration (µM)

Fig. 16
SUBSTITUTE SHEET (RULE 26)

cPKA BioST Forskolin dose-response on FLIPR (n=6).

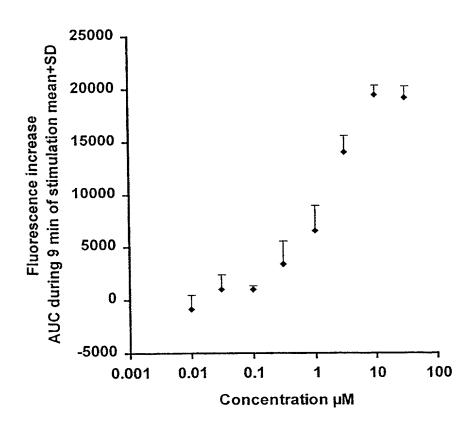


Fig. 17

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- 14.

18/18

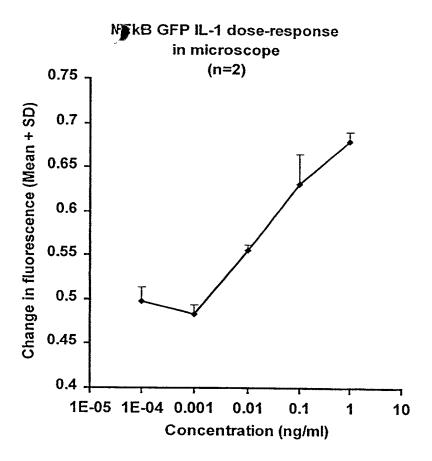


Fig. 18

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As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

nsert Title:	AN IMPROVED MET CELLULAR RESPONS		TRACTING QUANTITA	TIVE INFORMATION	N RELATING TO	AN INFLUE	NCE ON A						
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For Use Without			er			(:61: h1s)							
•	and amended on	use filed on Oct	ober 15, 1999			(п аррисавіе)	and/or as PCT						
Attacned:	International An	was med on <u>Oct</u>	or PCT/DK99/00562			; and was							
	amended under I		(if ap	olicable)									
	I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as												
7 (MIN).	amended by any amer I acknowledge the Regulations, \$1.56	ndment referred ne duty to discl	to above. lose information which is	material to patental	pility as defined in	Title 37, Coo	de of Federal						
	I do not know an	d do not believe	or used in the United	l States of America	before my or	our invention							
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	patent or inventor's co	ertificate on this	invention has been filed:	in any country foreign	n to the United Stat	tes of America	prior to this						
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	a filing date before tha	it of the applicat	ion on which priority is cl	aimed:									
4:500	Prior Foreign Appli	cation(s)				Priority C	laimed						
	DA 1009 01220	Donmark		October 15, 1998	R	\boxtimes							
	PA 1998 01320 (Number)	<u>Denmark</u> (Country)		(Month/Day/Yea		Yes	No						
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# 1000m	(Number)	(Country)		(Month/Day/Year	r Filed)	Yes	No						
	(Number)	(Country)	de war.	(Month/Day/Year	r Filed)	Yes	No						
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	(Number)	(Country)	.	(Month/Day/Yea	r Filed)	∐ Yes	∐ No						
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	Thereby claim the ber	ent under Tide (oo, crated outes code, gr	es(e) or any oracea on	ares provisional app	, included (b) 12.							
Insert Provisional Application(s): (if any)	(Application Number)	····	(Filing Date)									
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Insert Prior U.S.	****		***										
insert Provisional Applications (if appropriate) Insert Prior U.S. Application(s): (if any)	(Application Number)	(Filing Date)	(Sta	tus - patented, pend	ling, abandon	ed)						
Page 1 of 3	(Application Number)	(Filing Date)	(Sta	tus - patented, pend	ling, abandon	ed)						

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Inventor, if any: see above	Grith HAGEL	Carifle Hazel		280301										
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gac cc Asp Pr	c tct o Ser 35	cag Gln	aat Asn	aca Thr	gcc Ala	cag Gln 40	ttg Leu	gat Asp	cag Gln	ttt Phe	gat Asp 45	aga Arg	atc Ile	aag Lys	144
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gag ag Glu Se 65	t ggg r Gly	aac Asn	cac His	tac Tyr 70	gcc Ala	atg Met	aag Lys	atc Ile	tta Leu 75	gac Asp	aag Lys	cag Gln	aag Lys	gtg Val 80	240
gtg aa Val Ly	g cta s Leu	aag Lys	cag Gln 85	atc Ile	gag Glu	cac His	act Thr	ctg Leu 90	aat Asn	gag Glu	aag Lys	cgc Arg	atc Ile 95	ctg Leu	288
cag go Gln Al	c gtc a Val	aac Asn 100	ttc Phe	ccg Pro	ttc Phe	ctg Leu	gtc Val 105	aaa Lys	ctt Leu	gaa Glu	ttc Phe	tcc Ser 110	ttc Phe	aag Lys	336

gac Asp	aac Asn	tca Ser 115	aac Asn	ctg Leu	tac Tyr	atg Met	gtc Val 120	atg Met	gag Glu	tat Tyr	gta Val	gct Ala 125	ggt Gly	ggc Gly	gag Glu	384	ŀ
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gtc Val	aat Asn 290	Asp	atc Ile	aag Lys	aac Asn	cac His 295	Lys	tgg Trp	ttt Phe	gcc Ala	acç Thr	Thr	gac Asp	tgg Trp	att Ile	91	.2
gcc Ala 305	Il.e	: tat : Tyr	cag Gln	aga Arg	aag Lys 310	Val	gaa Glu	gct Ala	ccc Pro	tto Phe 315	: Ile	cca Pro	aag Lys	ttt Phe	aaa Lys 320	96	50
ggc Gly	cct Pro	ggg Gly	gac Asp	acg Thr	Ser	aac Asn	ttt Phe	gac Asp	gac Asp 330	Tyr	gaç Glu	gaç ıGlu	g gaa ı Glu	gaç Glu 335	atc lle	100)8

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cgc Arg	gcc Ala	atg Met 355	agt Ser	aaa Lys	gga Gly	gaa Glu	gaa Glu 360	ctt Leu	ttc Phe	act Thr	gga Gly	gtt Val 365	gtc Val	cca Pro	att Ile	1104
ctt Leu	gtt Val 370	gaa Glu	tta Leu	gat Asp	ggc Gly	gat Asp 375	gtt Val	aat Asn	ggg Gly	caa Gln	aaa Lys 380	ttc Phe	tct Ser	gtt Val	agt Ser	1152
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Gln Gln Gly Tyr Ile Gln Val Thr Asp Phe Gly Phe Ala Lys Arg Val 180 185 190

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Leu Leu Gln Val Asp Leu Thr Lys Arg Phe Gly Asn Leu Lys Asp Gly 275 280 285

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Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr 405 410 415

Thr Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met 420 425 430

Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln 435 $$ 445

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Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp 530 535 540

Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala 545 550 555 560

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Asp Pro Arg Ser Lys His Lys Phe Lys Ile His Thr Tyr Gly Ser Pro 100 105 110

Thr Phe Cys Asp His Cys Gly Ser Leu Leu Tyr Gly Leu Ile His Gln 115 $$120\,$

Gly Met Lys Cys Asp Thr Cys Asp Met Asn Val His Asn Gln Cys Val 130 135 140

Ile Asn Asp Pro Ser Leu Cys Gly Met Asp His Thr Glu Lys Arg Gly 145 150 155 160

Arg Ile Tyr Leu Lys Ala Glu Val Thr Asp Glu Lys Leu His Val Thr $165 \hspace{1.5cm} 170 \hspace{1.5cm} 175$

Val Arg Asp Ala Lys Asn Leu Ile Pro Met Asp Pro Asn Gly Leu Ser 180 185 190

Asp Pro Tyr Val Lys Leu Lys Leu Ile Pro Asp Pro Lys Asn Glu Ser 195 200 205

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Glu Ser Phe Thr Phe Lys Leu Lys Pro Ser Asp Lys Asp Arg Arg Leu 225 230 235 235

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765

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gcc cga aac tac Ala Arg Asn Tyr 530	cta cag tct Leu Gln Ser 535	ctg ccc tcc Leu Pro Ser	aag acc aag gtg Lys Thr Lys Val 540	gct tgg 1632 Ala Trp
			gcc ctt gac ctg Ala Leu Asp Leu 555	
			atc aca gtg gag Ile Thr Val Glu	
			gac ccg acg gat Asp Pro Thr Asp 590	
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Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe 210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser 225 230 235 240

Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Thr Met Ala Ala Ala 245 250 255

Ala Ala Gl
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Gly Pro Gly Val Pro Gly Glu Val Glu Met Val Lys Gly Gln Pro Phe 275 280 285

Asp Val Gly Pro Arg Tyr Thr Gln Leu Gln Tyr Ile Gly Glu Gly Ala 290 295 300

Tyr Gly Met Val Ser Ser Ala Tyr Asp His Val Arg Lys Thr Arg Val 305 310 315 320

Ala Ile Lys Lys Ile Ser Pro Phe Glu His Gln Thr Tyr Cys Gln Arg 325 330 335

Thr Leu Arg Glu Ile Gln Ile Leu Leu Arg Phe Arg His Glu Asn Val 340 345 350

Ile Gly Ile Arg Asp Ile Leu Arg Ala Ser Thr Leu Glu Ala Met Arg 355 360 365

Asp Val Tyr Ile Val Gln Asp Leu Met Glu Thr Asp Leu Tyr Lys Leu 370 375 380

Leu Lys Ser Gln Gln Leu Ser Asn Asp His Ile Cys Tyr Phe Leu Tyr 385 390 395 400

Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asn Val Leu His 405 410 415

Arg Asp Leu Lys Pro Ser Asn Leu Leu Ser Asn Thr Thr Cys Asp Leu 420 425 430

Lys Ile Cys Asp Phe Gly Leu Ala Arg Ile Ala Asp Pro Glu His Asp 435 440 445

Pro Glu Ile Met Leu Asn Ser Lys Gly Tyr Thr Lys Ser Ile Asp Ile 465 470 475 480

Trp Ser Val Gly Cys Ile Leu Ala Glu Met Leu Ser Asn Arg Pro Ile 485 490 495

Phe Pro Gly Lys His Tyr Leu Asp Gln Leu Asn His Ile Leu Gly Ile 500 505 510

Leu Gly Ser Pro Ser Gln Glu Asp Leu Asn Cys Ile Ile Asn Met Lys 515 520 525

Ala Arg Asn Tyr Leu Gln Ser Leu Pro Ser Lys Thr Lys Val Ala Trp 530 535 540

Ala Lys Leu Phe Pro Lys Ser Asp Ser Lys Ala Leu Asp Leu Leu Asp 545 550 560

Arg Met Leu Thr Phe Asn Pro Asn Lys Arg Ile Thr Val Glu Glu Ala 565 570 575

Leu Ala His Pro Tyr Leu Glu Gln Tyr Tyr Asp Pro Thr Asp Glu Pro 580 585 590

Val Ala Glu Glu Pro Phe Thr Phe Ala Met Glu Leu Asp Asp Leu Pro 595 600 605

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gag Glu	ggc Gly	gag Glu 35	ggc Gly	gat Asp	gcc Ala	acc Thr	tac Tyr 40	ggc Gly	aag Lys	ctg Leu	acc Thr	ctg Leu 45	aag Lys	ttc Phe	atc Ile	144
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tct Ser	cca Pro	gca Ala	gaa Glu 500	cta Leu	tct Ser	cct Pro	act Thr	act Thr 505	ctt Leu	tcc Ser	cct Pro	gtt Val	aat Asn 510	cat His	agc Ser		1536
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ata Ile	gca Ala 530	tat Tyr	tat Tyr	gaa Glu	tta Leu	aat Asn 535	cag Gln	agg Arg	gtt Val	gga Gly	gaa Glu 540	acc Thr	ttc Phe	cat His	gca Ala		1632
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130

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135

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn 150 145 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser 170 165 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly 180 185 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu 200 195 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe 215 220 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser 235 230 Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Thr Met Ser Ser Ile 245 250 Leu Pro Phe Thr Pro Pro Val Val Lys Arg Leu Leu Gly Trp Lys Lys 260 265 Ser Ala Gly Gly Ser Gly Gly Ala Gly Gly Glu Gln Asn Gly Gln 280 275 Glu Glu Lys Trp Cys Glu Lys Ala Val Lys Ser Leu Val Lys Lys Leu 295 300 290 Lys Lys Thr Gly Arg Leu Asp Glu Leu Glu Lys Ala Ile Thr Thr Gln 310 315 320 305 Asn Cys Asn Thr Lys Cys Val Thr Ile Pro Ser Thr Cys Ser Glu Ile 330 325 Trp Gly Leu Ser Thr Pro Asn Thr Ile Asp Gln Trp Asp Thr Thr Gly 345 340

Leu Tyr Ser Phe Ser Glu Gln Thr Arg Ser Leu Asp Gly Arg Leu Gln 360

Val Ser His Arg Lys Gly Leu Pro His Val Ile Tyr Cys Arg Leu Trp 370 375 380

Arg Trp Pro Asp Leu His Ser His His Glu Leu Lys Ala Ile Glu Asn 385 390 395 400

Cys Glu Tyr Ala Phe Asn Leu Lys Lys Asp Glu Val Cys Val Asn Pro $405 \hspace{1cm} 410 \hspace{1cm} 415 \hspace{1cm}$

Tyr His Tyr Gln Arg Val Glu Thr Pro Val Leu Pro Pro Val Leu Val 420 425 430

Pro Arg His Thr Glu Ile Leu Thr Glu Leu Pro Pro Leu Asp Asp Tyr 435 440 445

Gln Ser Asn Tyr Ile Pro Glu Thr Pro Pro Pro Gly Tyr Ile Ser Glu 465 470 475 480

Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser Met Asp Thr Gly 485 490 495

Ser Pro Ala Glu Leu Ser Pro Thr Thr Leu Ser Pro Val Asn His Ser 500 505 510

Leu Asp Leu Gln Pro Val Thr Tyr Ser Glu Pro Ala Phe Trp Cys Ser 515 520 525

Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu Thr Phe His Ala 530 535 540

Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp Pro Ser Asn Ser 545 550 555 560

Glu Arg Phe Cys Leu Gly Leu Leu Ser Asn Val Asn Arg Asn Ala Thr 565 570 575

Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val Arg Leu Tyr Tyr 580 585 590

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tgc Cys	tct Ser	gaa Glu	att Ile	tgg Trp 85	gga Gly	ctg Leu	agt Ser	aca Thr	cca Pro 90	aat Asn	acg Thr	ata Ile	gat Asp	cag Gln 95	tgg Trp	288
gat Asp	aca Thr	aca Thr	ggc Gly 100	ctt Leu	tac Tyr	agc Ser	ttc Phe	tct Ser 105	gaa Glu	caa Gln	acc Thr	agg Arg	tct Ser 110	ctt Leu	gat Asp	336
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Pro	Val	Leu	Val 180	Pro	Arg	His	Thr	Glu 185		Leu	Thr	Glu	Leu 190	Pro	Pro	576
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									cca Pro			Pro				672
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				Leu					Val					Pro	gca Ala	816

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cca Pro 305	tca Ser	aat Asn	tca Ser	gag Glu	agg Arg 310	ttc Phe	tgc Cys	tta Leu	ggt Gly	tta Leu 315	ctc Leu	tcc Ser	aat Asn	gtt Val	aac Asn 320	960
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Asp	Phe 610	Lys	Glu	Asp	ggc Gly	Asn 615	Ile	Leu	Gly	His	Lys 620	Leu	Glu	Tyr	Asn	1872
Tyr 625	Asn	Ser	His	Asn	gtc Val 630	Tyr	Ile	Met	Ala	Asp 635	Lys	Gln	Lys	Asn	Gly 640	1920
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				His	tac Tyr				Thr					Gly		2016
gtg Val	ctg Leu	ctg Leu 675	Pro	gac Asp	aac Asn	cac His	tac Tyr 680	Leu	agc Ser	acc Thr	cag Gln	Ser 685	Ala	ctg Leu	agc Ser	2064
aaa Lys	gac Asp 690	Pro	aac Asn	gag Glu	aag Lys	cgc Arg 695	Asp	cac His	atg Met	gtc Val	ctg Leu 700	Leu	gag Glu	tto Phe	gtg Val	2112
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Gln Asn Gly Gln Glu Glu Lys Trp Cys Glu Lys Ala Val Lys Ser Leu 35 40 45

Val Lys Lys Leu Lys Lys Thr Gly Arg Leu Asp Glu Leu Glu Lys Ala 50 55 60

Ile Thr Thr Gln Asn Cys Asn Thr Lys Cys Val Thr Ile Pro Ser Thr
65 70 75 80

Cys Ser Glu Ile Trp Gly Leu Ser Thr Pro Asn Thr Ile Asp Gln Trp 85 90 95

Asp Thr Thr Gly Leu Tyr Ser Phe Ser Glu Gln Thr Arg Ser Leu Asp 100 105 110

Gly Arg Leu Gln Val Ser His Arg Lys Gly Leu Pro His Val Ile Tyr 115 120 125

Cys Arg Leu Trp Arg Trp Pro Asp Leu His Ser His His Glu Leu Lys 130 135 140

Ala Ile Glu Asn Cys Glu Tyr Ala Phe Asn Leu Lys Lys Asp Glu Val 145 150 155 160

Cys Val Asn Pro Tyr His Tyr Gln Arg Val Glu Thr Pro Val Leu Pro
165 170 175

Pro Val Leu Val Pro Arg His Thr Glu Ile Leu Thr Glu Leu Pro Pro 180 185 190

Leu Asp Asp Tyr Thr His Ser Ile Pro Glu Asn Thr Asn Phe Pro Ala 195 200 205

Gly Ile Glu Pro Gln Ser Asn Tyr Ile Pro Glu Thr Pro Pro Pro Gly 210 215 220

Tyr Ile Ser Glu Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser 225 230 235 240

Met Asp Thr Gly Ser Pro Ala Glu Leu Ser Pro Thr Thr Leu Ser Pro 245 250

Val Asn His Ser Leu Asp Leu Gln Pro Val Thr Tyr Ser Glu Pro Ala 260 265 270

Phe Trp Cys Ser Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu 275 280 285

Thr Phe His Ala Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp 290 295 300

Pro Ser Asn Ser Glu Arg Phe Cys Leu Gly Leu Leu Ser Asn Val Asn 305 310 315 320

Arg Asn Ala Thr Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val 325 330 335

Arg Leu Tyr Tyr Ile Gly Gly Glu Val Phe Ala Glu Cys Leu Ser Asp 340 345 350

Ser Ala Ile Phe Val Gln Ser Pro Asn Cys Asn Gln Arg Tyr Gly Trp 355 360 365

His Pro Ala Thr Val Cys Lys Ile Pro Pro Gly Cys Asn Leu Lys Ile 370 375 380

Phe Asn Asn Gln Glu Phe Ala Ala Leu Leu Ala Gln Ser Val Asn Gln 385 390 395 400

Gly Phe Glu Ala Val Tyr Gln Leu Thr Arg Met Cys Thr Ile Arg Met $405 \hspace{1.5cm} 410 \hspace{1.5cm} 415 \hspace{1.5cm}$

Ser Thr Pro Cys Trp Ile Glu Leu His Leu Asn Gly Pro Leu Gln Trp $435 \hspace{1.5cm} 440 \hspace{1.5cm} 445$

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Ser Met Ser Trp Val Pro Arg Ala Arg Asp Pro Pro Val Ala Thr Met 465 470 475 480

Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val 485 490 495

Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu 500 505 510

Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys 515 520 525

Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu 530 535 540

Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln 545 550 555 560

His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg 565 570 575

Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val 580 585 590

Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile 595 600 605

Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn 610 615 620

Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly 625 630 635 640

Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val 645 650 655	
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro 660 665 670	
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser 675 680 685	
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gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile 35 40 45	144
tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr 50 55 60	192
ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys 65 70 75 80	240
cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag	288

													cgc Arg 110			336
gtg Val	aag Lys	ttc Phe 115	gag Glu	ggc Gly	gac Asp	acc Thr	ctg Leu 120	gtg Val	aac Asn	cgc Arg	atc Ile	gag Glu 125	ctg Leu	aag Lys	ggc Gly	384
atc Ile	gac Asp 130	ttc Phe	aag Lys	gag Glu	gac Asp	ggc Gly 135	aac Asn	atc Ile	ctg Leu	ggg Gly	cac His 140	aag Lys	ctg Leu	gag Glu	tac Tyr	432
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ccc Pro	gtg Val	ctg Leu 195	ctg Leu	ccc Pro	gac Asp	aac Asn	cac His 200	tac Tyr	ctg Leu	agc Ser	acc Thr	cag Gln 205	tcc Ser	gcc Ala	ctg Leu	624
agc Ser	aaa Lys 210	gac Asp	ccc Pro	aac Asn	gag Glu	aag Lys 215	cgc Arg	gat Asp	cac His	atg Met	gtc Val 220	ctg Leu	ctg Leu	gag Glu	ttc Phe	672
													tac Tyr			720
													gtc Val			768
													ctt Leu 270			816
								Ala					cag Gln			864
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	Gly										Val				gcc Ala 320	960

atc Ile	gtc Val	cgg Arg	ggt Gly	gtc Val 325	aag Lys	tat Tyr	aac Asn	cag Gln	gcc Ala 330	acc Thr	ccc Pro	aac Asn	ttc Phe	cat His 335	cag Gln	1008
								ggc Gly 345								1056
gat Asp	gcg Ala	gcc Ala 355	cag Gln	ttt Phe	gcc Ala	gcc Ala	ggc Gly 360	atg Met	gcc Ala	agt Ser	gcc Ala	cta Leu 365	gag Glu	gcg Ala	ttg Leu	1104
gaa Glu	gga Gly 370	ggt Gly	ggg Gly	ccc Pro	cct Pro	cca Pro 375	ccc Pro	cca Pro	gca Ala	ctt Leu	ccc Pro 380	acc Thr	tgg Trp	tcg Ser	gtc Val	1152
ccg Pro 385	aac Asn	ggc Gly	ccc Pro	tcc Ser	ccg Pro 390	gag Glu	gag Glu	gtg Val	gag Glu	cag Gln 395	cag Gln	aaa Lys	agg Arg	cag Gln	cag Gln 400	1200
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								cca Pro 425								1296
cct Pro	cct Pro	cca Pro 435	ggt Gly	ccc Pro	ccc Pro	cca Pro	ccc Pro 440	cca Pro	ggt Gly	ttg Leu	ccc Pro	cct Pro 445	tcg Ser	Gly	gtc Val	1344
cca Pro	gct Ala 450	gca Ala	gcg Ala	cac His	gga Gly	gca Ala 455	Gly	gga Gly	gga Gly	cca Pro	ccc Pro 460	Pro	gca Ala	ccc Pro	cct Pro	1392
															ggc Gly 480	1440
Leu	Āla	Āla	Ala	Ile 485	Ala	Gly	Ala	Lys	Leu 490	Arg	Lys	Val	Ser	Lys 495		1488
gag Glu	gag Glu	gcc Ala	tca Ser 500	Gly	gly	ccc Pro	aca Thr	gcc Ala 505	Pro	aaa Lys	gct Ala	gag Glu	agt Ser 510	ggt Gly	cga Arg	1536
agc Ser	gga Gly	ggt Gly 515	Gly	gga Gly	ctc Leu	atg Met	gaa Glu 520	Glu	atg Met	aac Asn	gcc Ala	atg Met 525	Leu	gcc Ala	cgg Arg	1584
aga Arg	agg Arg 530	Lys	gcc Ala	acg Thr	caa Gln	gtt Val 535	Gly	gag Glu	aaa Lys	acc Thr	ecc Pro 540	Lys	gat Asp	gaa Glu	tct Ser	1632

Ala Asn Gln Glu Glu Pro Glu Ala Arg Val Pro Ala Gln Ser Glu Ser 545 550 550 560	1680
gtg cgg aga ccc tgg gag aag aac agc aca acc ttg cca agg atg aag Val Arg Arg Pro Trp Glu Lys Asn Ser Thr Thr Leu Pro Arg Met Lys 565 570 575	1728
tcg tct tct tcg gtg acc act tcc gag acc caa ccc tgc acg ccc agc Ser Ser Ser Ser Val Thr Thr Ser Glu Thr Gln Pro Cys Thr Pro Ser 580 585 590	1776
tcc agt gat tac tcg gac cta cag agg gtg aaa cag gag ctt ctg gaa Ser Ser Asp Tyr Ser Asp Leu Gln Arg Val Lys Gln Glu Leu Leu Glu 595 600 605	1824
gag gtg aag aag gaa ttg cag aaa gtg aaa gag gaa atc att gaa gcc Glu Val Lys Lys Glu Leu Gln Lys Val Lys Glu Glu Ile Ile Glu Ala 610 615 620	1872
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Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser \$165\$ \$170\$ \$175\$

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly $180 \,$ $185 \,$ 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe 210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser 225 230 235 240

Gly Leu Arg Ser Arg Ala Gln Ala Ser Met Ser Glu Thr Val Ile Met 245 250 255

Ser Glu Thr Val Ile Cys Ser Ser Arg Ala Thr Val Met Leu Tyr Asp 260 265 270

Asp Gly Asn Lys Arg Trp Leu Pro Ala Gly Thr Gly Pro Gln Ala Phe 275 280 285

Ser Arg Val Gln Ile Tyr His Asn Pro Thr Ala Asn Ser Phe Arg Val 290 295 300

Val Gly Arg Lys Met Gln Pro Asp Gln Gln Val Val Ile Asn Cys Ala 305 310315315320

Ile Val Arg Gly Val Lys Tyr Asn Gln Ala Thr Pro Asn Phe His Gln \$325\$ \$330 \$35

Trp Arg Asp Ala Arg Gln Val Trp Gly Leu Asn Phe Gly Ser Lys Glu 340 345 350

Asp Ala Gln Phe Ala Ala Gly Met Ala Ser Ala Leu Glu Ala Leu 355 360 365

Glu Gly Gly Gly Pro Pro Pro Pro Pro Ala Leu Pro Thr Trp Ser Val 370 375 380

Pro Asn Gly Pro Ser Pro Glu Glu Val Glu Gln Gln Lys Arg Gln Gln 385 390 395 400

Pro Gly Pro Ser Glu His Ile Glu Arg Arg Val Ser Asn Ala Gly Gly \$405\$

Pro Pro Ala Pro Pro Ala Gly Gly Pro Pro Pro Pro Pro Gly Pro Pro 420 425 430

Pro Pro Pro Gly Pro Pro Pro Pro Pro Gly Leu Pro Pro Ser Gly Val435 440 445

Pro Ala Ala Ala His Gly Ala Gly Gly Gly Pro Pro Ala Pro Pro 450 455 460

Leu Pro Ala Ala Gln Gly Pro Gly Gly Gly Gly Ala Gly Ala Pro Gly 465 470 475 480

Leu Ala Ala Ala Ile Ala Gly Ala Lys Leu Arg Lys Val Ser Lys Gln 485 490 495

Glu Glu Ala Ser Gly Gly Pro Thr Ala Pro Lys Ala Glu Ser Gly Arg 500 505 510

Ser Gly Gly Gly Leu Met Glu Glu Met Asn Ala Met Leu Ala Arg 515 520 525

Arg Arg Lys Ala Thr Gln Val Gly Glu Lys Thr Pro Lys Asp Glu Ser 530 540

	Glu Glu	550	Ala Arg	Val Pro 555	Ala Gln	Ser Glu	Ser 560
Val Arg Arg	Pro Trp 565	Glu Lys	Asn Ser	Thr Thr 570	Leu Pro	Arg Met 575	
Ser Ser Ser	Ser Val 580	Thr Thr	Ser Glu 585	Thr Gln	Pro Cys	Thr Pro	Ser
Ser Ser Asp 595	-	Asp Leu	Gln Arg 600	Val Lys	Gln Glu 605	Leu Leu	Glu
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atg gtg ago Met Val Ser 1 gtc gag ctg	Lys Gly 5 gac ggc Asp Gly 20 ggc gat	gac gta Asp Val	Leu Phe aac ggc Asn Gly 25 tac ggc	Thr Gly 10 cac aag His Lys aag ctg	ttc ago Phe Ser	Pro Ile 15 gtg tcc Val Sen 30	e Leu e ggc 96 c Gly c atc 144
atg gtg ago Met Val Ser 1 gtc gag ctg Val Glu Leu gag ggc gag Glu Gly Glu	Lys Gly 5 gac ggc Asp Gly 20 ggc gat Gly Asp	gac gta Asp Val gcc acc Ala Thr	Leu Phe aac ggc Asn Gly 25 tac ggc Tyr Gly 40 gtg ccc	Thr Gly 10 cac aag His Lys aag ctg Lys Leu tgg ccc	ttc ago Phe Ser acc ctg Thr Leu 45 acc ctc	Pro Ile 15 gtg tcc Val Ser 30 aag ttc Lys Phe	e Leu e ggc 96 e Gly c atc 144 e Ile c acc 192

				aag Lys						288
				aag Lys						336
				gac Asp						384
				gac Asp						432
				aac Asn 150						480
				ttc Phe						528
				cac His						576
				gac Asp						624
				gag Glu						672
	_	_		atc Ile 230						720
				gcc Ala						768
_		_	_	gcc Ala						816
				atg Met						864
				ggc Gly						912

							aca Thr					960
	_	-		_	_		cac His					1008
							ttc Phe 345					1056
							aac Asn					1104
							agt Ser					1152
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	_				_	_	gtg Val					1248
							ctt Leu 425					1296
							aag Lys					1344
							gag Glu					1392
							tat Tyr					1440
							gat Asp					1488
							gac Asp 505					1536
_	_		_	_	_		cct Pro					1584

					tac Tyr											1632
					agg Arg 550											1680
					gga Gly											1728
	_				cgc Arg	_		_								1776
_					acg Thr			_	_					_		1824
					ttt Phe											1872
		_	_		ccc Pro 630		_	_		_	_				_	1920
					gta Val											1968
	-		-		ggc Gly			_	-		-			_		2016
_			_	_	Gly	_		_	_			-	_	_	_	2064
_	_		_	_	gaa Glu	_	-		_	_				-		2112
_		_			aca Thr 710	-	_	_		_	_					2160
_	_	_	_		cag Gln					_						2208
					tac Tyr											2256

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Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr 50 55 60	
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys 65 70 75 80	
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu 85 90 95	
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu 100 105 110	
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly 115 120 125	
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr 130 135 140	

Asn 145	Tyr	Asn	Ser	His	Asn 150	Val	Tyr	Ile	Met	Ala 155	Asp	Lys	Gln	Lys	Asn 160
Gly	Ile	Lys	Val	Asn 165	Phe	Lys	Ile	Arg	His 170	Asn	Ile	Glu	Asp	Gly 175	Ser
Val	Gln	Leu	Ala 180	Asp	His	Tyr	Gln	Gln 185	Asn	Thr	Pro	Ile	Gly 190	Asp	Gly
Pro	Val	Leu 195	Leu	Pro	Asp	Asn	His 200	Tyr	Leu	Ser	Thr	Gln 205	Ser	Ala	Leu
Ser	Lys 210	Asp	Pro	Asn	Glu	Lys 215	Arg	Asp	His	Met	Val 220	Leu	Leu	Glu	Phe
Val 225	Thr	Ala	Ala	Gly	Ile 230	Thr	Leu	Gly	Met	Asp 235	Glu	Leu	Tyr	Lys	Ser 240
Gly	Leu	Arg	Ser	Arg 245	Ala	Met	Asp	Glu	Leu 250	Phe	Pro	Leu	Ile	Phe 255	Pro
Ala	Glu	Pro	Ala 260	Gln	Ala	Ser	Gly	Pro 265	Tyr	Val	Glu	Ile	Ile 270	Glu	Gln
Pro	Lys	Gln 275	Arg	Gly	Met	Arg	Phe 280	Arg	Tyr	Lys	Cys	Glu 285	Gly	Arg	Ser
Ala	Gly 290	Ser	Ile	Pro	Gly	Glu 295	Arg	Ser	Thr	Asp	Thr 300	Thr	Lys	Thr	His
Pro 305	Thr	Ile	Lys	Ile	Asn 310	Gly	Tyr	Thr	Gly	Pro 315	Gly	Thr	Val	Arg	Ile 320
Ser	Leu	Val	Thr	Lys 325	Asp	Pro	Pro	His	Arg 330	Pro	His	Pro	His	Glu 335	Leu
Val	Gly	Lys	Asp 340	Cys	Arg	Asp	Gly	Phe 345	Tyr	Glu	Ala	Glu	Leu 350	Cys	Pro
Asp	Arg	Cys 355	Ile	His	Ser	Phe	Gln 360	Asn	Leu	Gly	Ile	Gln 365	Cys	Val	Lys

Lys	Arg 370	Asp	Leu	Glu	Gln	Ala 375	Ile	Ser	Gln	Arg	Ile 380	Gln	Thr	Asn	Asn
Asn 385	Pro	Phe	Gln	Val	Pro 390	Ile	Glu	Glu	Gln	Arg 395	Gly	Asp	Tyr	Asp	Leu 400
Asn	Ala	Val	Arg	Leu 405	Cys	Phe	Gln	Val	Thr 410	Val	Arg	Asp	Pro	Ser 415	Gly
Arg	Pro	Leu	Arg 420	Leu	Pro	Pro	Val	Leu 425	Pro	His	Pro	Ile	Phe 430	Asp	Asn
Arg	Ala	Pro 435	Asn	Thr	Ala	Glu	Leu 440	Lys	Ile	Cys	Arg	Val 4 4 5	Asn	Arg	Asn
Ser	Gly 450	Ser	Cys	Leu	Gly	Gly 455	Asp	Glu	Ile	Phe	Leu 460	Leu	Cys	Asp	Lys
Val 465	Gln	Lys	Glu	Asp	Ile 470	Glu	Val	Tyr	Phe	Thr 475	Gly	Pro	Gly	Trp	Glu 480
Ala	Arg	Gly	Ser	Phe 485	Ser	Gln	Ala	Asp	Val 490	His	Arg	Gln	Val	Ala 495	Ile
Val	Phe	Arg	Thr 500	Pro	Pro	Tyr	Ala	Asp 505	Pro	Ser	Leu	Gln	Ala 510	Pro	Val
Arg	Val	Ser 515	Met	Gln	Leu	Arg	Arg 520	Pro	Ser	Asp	Arg	Glu 525	Leu	Ser	Glu
Pro	Met 530	Glu	Phe	Gln	Tyr	Leu 535	Pro	Asp	Thr	Asp	Asp 540	Arg	His	Arg	Ile
Glu 545	Glu	Lys	Arg	Lys	Arg 550	Thr	Tyr	Glu	Thr	Phe 555	Lys	Ser	Ile	Met	Lys 560

Ile Ala Val Pro Ser Arg Ser Ser Ala Ser Val Pro Lys Pro Ala Pro 580 585 590

Lys Ser Pro Phe Ser Gly Pro Thr Asp Pro Arg Pro Pro Pro Arg Arg 565 575

Gln Pro Tyr Pro Phe Thr Ser Ser Leu Ser Thr Ile Asn Tyr Asp Glu 595 600

Phe Pro Thr Met Val Phe Pro Ser Gly Gln Ile Ser Gln Ala Ser Ala 610 615 620

Leu Ala Pro Ala Pro Pro Gln Val Leu Pro Gln Ala Pro Ala Pro Ala 625 630 635 640

Pro Ala Pro Ala Met Val Ser Ala Leu Ala Gln Ala Pro Ala Pro Val 645 650 655

Pro Val Leu Ala Pro Ġly Pro Pro Gln Ala Val Ala Pro Pro Ala Pro 660 665 670

Lys Pro Thr Gln Ala Gly Glu Gly Thr Leu Ser Glu Ala Leu Leu Gln 675 680 685

Leu Gln Phe Asp Asp Glu Asp Leu Gly Ala Leu Leu Gly Asn Ser Thr 690 695 700

Asp Pro Ala Val Phe Thr Asp Leu Ala Ser Val Asp Asn Ser Glu Phe 705 710 715 720

Gln Gln Leu Leu Asn Gln Gly Ile Pro Val Ala Pro His Thr Thr Glu 725 730 735

Pro Met Leu Met Glu Tyr Pro Glu Ala Ile Thr Arg Leu Val Thr Gly $740 \hspace{1cm} 745 \hspace{1cm} 750$

Ala Gln Arg Pro Pro Asp Pro Ala Pro Ala Pro Leu Gly Ala Pro Gly 755 760 765

Leu Pro Asn Gly Leu Leu Ser Gly Asp Glu Asp Phe Ser Ser Ile Ala 770 785

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		ctt gta gga aag gad Leu Val Gly Lys Asp 90	
		ccg gac cgc tgc ato Pro Asp Arg Cys Ile 110	His Ser
	2 2 2 2	aag aag cgg gac ctg Lys Lys Arg Asp Leu 125	
		aac aac ccc ttc caa Asn Asn Pro Phe Glr 140	
		ctg aat gct gtg cgg Leu Asn Ala Val Arg 155	_
	Arg Asp Pro Ser	ggc agg ccc ctc cgc Gly Arg Pro Leu Arc 170	
-		aat cgt gcc ccc aac Asn Arg Ala Pro Asr 190	Thr Ala

					cga Arg											624
	_				cta Leu	_	-	-								672
				-	gga Gly 230					-	_				_	720
	-	-			cga Arg			_								768
	_	_		_	ctg Leu	_	-			_	_		_	_	_	816
					cgg Arg											864
					gat Asp											912
					aag Lys 310	_		_	_	_	_			_		960
		_			cct Pro			-	_		_				_	1008
_				_	ccc Pro	_		_		_					_	1056
		_	_		atc Ile			_					_			1104
			-		agc Ser	_	_	_	_	_	_	_	_			1152
					gct Ala 390											1200
	_	_	_	_	gcc Ala		_		_		_		-			1248

					gcc Ala											1296
_		_	_		gag Glu	-	_	_	_	_	_		-		-	1344
					ctt Leu											1392
_	_	_		_	gac Asp 470					_	_	_	_			1440
					ccc Pro											1488
					cgc Arg											1536
					ctg Leu											1584
		-	_	_	ttc Phe					_	_	_			-	1632
					agc Ser 550											1680
					ctg Leu											1728
_	_		_	_	aac Asn			_								1776
					tac Tyr											1824
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			_	_	ttc Phe 630	_				_		_	_	_		1920

gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag cgc Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg 645 650 655	
atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val 660 665 670	
ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile 675 680 685	_
ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac aac Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn 690 695 700	
aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac ggc Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly 705 710 715	
aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc gtg Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val 725 730 735	
ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc ccc Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro 740 745 750	
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gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val 770 775 780	
gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag taa Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys 785 790 795	2394
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Glu Arg Ser Thr Asp Thr Thr Lys Thr His Pro Thr Ile Lys Ile Asn 50 55 60

Gly Tyr Thr Gly Pro Gly Thr Val Arg Ile Ser Leu Val Thr Lys Asp 65 70 75 80

Pro Pro His Arg Pro His Pro His Glu Leu Val Gly Lys Asp Cys Arg 85 90 95

Asp Gly Phe Tyr Glu Ala Glu Leu Cys Pro Asp Arg Cys Ile His Ser 100 105 110

Phe Gln Asn Leu Gly Ile Gln Cys Val Lys Lys Arg Asp Leu Glu Gln 115 120 125

Ala Ile Ser Gln Arg Ile Gln Thr Asn Asn Asn Pro Phe Gln Val Pro 130 135 140

Ile Glu Glu Gln Arg Gly Asp Tyr Asp Leu Asn Ala Val Arg Leu Cys 145 150 155 160

Phe Gln Val Thr Val Arg Asp Pro Ser Gly Arg Pro Leu Arg Leu Pro 165 170 175

Pro Val Leu Pro His Pro Ile Phe Asp Asn Arg Ala Pro Asn Thr Ala 180 185 190

Glu Leu Lys Ile Cys Arg Val Asn Arg Asn Ser Gly Ser Cys Leu Gly
195 200 205

Gly Asp Glu Ile Phe Leu Cys Asp Lys Val Gln Lys Glu Asp Ile 210 215 220

Glu Val Tyr Phe Thr Gly Pro Gly Trp Glu Ala Arg Gly Ser Phe Ser 225 230 235 240

Gln Ala Asp Val His Arg Gln Val Ala Ile Val Phe Arg Thr Pro Pro $245 \\ \hspace{1.5cm} 250 \\ \hspace{1.5cm} 255$

Tyr	Ala	Asp	Pro	Ser	Leu	Gln	Ala	Pro	Val	Arg	Val	Ser	Met	Gln	Leu
_		_	260					265					270		

Arg Arg Pro Ser Asp Arg Glu Leu Ser Glu Pro Met Glu Phe Gln Tyr 275 280 285

Leu Pro Asp Thr Asp Asp Arg His Arg Ile Glu Glu Lys Arg Lys Arg 290 295 300

Thr Tyr Glu Thr Phe Lys Ser Ile Met Lys Lys Ser Pro Phe Ser Gly 305 310 315 320

Pro Thr Asp Pro Arg Pro Pro Pro Arg Arg Ile Ala Val Pro Ser Arg 325 330 335

Ser Ser Ala Ser Val Pro Lys Pro Ala Pro Gln Pro Tyr Pro Phe Thr 340 345 350

Ser Ser Leu Ser Thr Ile Asn Tyr Asp Glu Phe Pro Thr Met Val Phe 355 360 365

Pro Ser Gly Gln Ile Ser Gln Ala Ser Ala Leu Ala Pro Ala Pro Pro 370 375 380

Gln Val Leu Pro Gln Ala Pro Ala Pro Ala Pro Ala Pro Ala Met Val 385 390 395 400

Ser Ala Leu Ala Gl
n Ala Pro Ala Pro Val Pro Val Leu Ala Pro Gly $405 \hspace{1cm} 410 \hspace{1cm} 415$

Pro Pro Gln Ala Val Ala Pro Pro Ala Pro Lys Pro Thr Gln Ala Gly
420 425 430

Glu Gly Thr Leu Ser Glu Ala Leu Leu Gln Leu Gln Phe Asp Asp Glu 435 440 445

Asp Leu Gly Ala Leu Leu Gly Asn Ser Thr Asp Pro Ala Val Phe Thr 450 455 460

Asp Leu Ala Ser Val Asp Asn Ser Glu Phe Gln Gln Leu Leu Asn Gln 465 470 475 480

Gly Ile Pro Val Ala Pro His Thr Thr Glu Pro Met Leu Met Glu Tyr 485 490 495

Pro Glu Ala Ile Thr Arg Leu Val Thr Gly Ala Gln Arg Pro Pro Asp 500 505 510

Pro Ala Pro Ala Pro Leu Gly Ala Pro Gly Leu Pro Asn Gly Leu Leu 515 520 525

Ser Gly Asp Glu Asp Phe Ser Ser Ile Ala Asp Met Asp Phe Ser Ala 530 540

Leu Leu Ser Gln Ile Ser Ser Leu Asp Pro Pro Val Ala Thr Met Val 545 550 550 560

Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu 565 570 575

Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly 580 585 590

Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr 595 600 605

Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr $610 \\ \hspace{1.5cm} 615 \\ \hspace{1.5cm} 620 \\ \hspace{1.5cm}$

Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His 625 630 635 640

Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr 645 650 655

Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys 660 665 670

Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp 675 680 685

Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr 690 695 700

705	Asn Val	Tyr Ile 710	Met Ala		Lys Gln 715	Lys Asn	Gly	11e 720	
Lys Val Asn	Phe Lys 725	Ile Arg	His Asr	n Ile G 730	Glu Asp	Gly Ser	Val 735	Gln	
Leu Ala Asp	His Tyr 740	Gln Gln	Asn Thi		Ile Gly	Asp Gly 750	Pro	Val	
Leu Leu Pro 755	Asp Asn	His Tyr	Leu Sei 760	r Thr G	Gln Ser	Ala Leu 765	Ser	Lys	
Asp Pro Asn 770	Glu Lys	Arg Asp 775	His Met	t Val I	Leu Leu 780	Glu Phe	Val	Thr	
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atg gtg agc Met Val Ser 1 gtc gag ctg	Lys Gly 5 gac ggc Asp Gly 20 ggc gat	Glu Glu gac gta Asp Val gcc acc	Leu Ph aac gg Asn Gl 25 tac gg	e Thr (10 c cac a y His I	Gly Val aag ttc Lys Phe ctg acc	val Pro agc gtg Ser Val 30 ctg aag	Ile 15 tcc Ser	Leu ggc Gly atc 1	
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					aag Lys								288
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					cac His								576
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_			_	_	ccc Pro			_	_	_	-		912

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					cac His								1152
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, ,,		atc aaa gag cat gca ttt Ele Lys Glu His Ala Phe 845	
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		acc ccc act gat aaa cto Thr Pro Thr Asp Lys Leu 890	
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Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr 50 60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly $115 \\ 120 \\ 125$

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser \$165\$ \$170\$ \$175\$

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
180 185 190

Pro Val Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe 210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser 225 230 235 240

Gly Leu Arg Ser Arg Gly Lys Met Ala Asp Pro Ala Ala Gly Pro Pro 245 250 255

Pro Ser Glu Gly Glu Glu Ser Thr Val Arg Phe Ala Arg Lys Gly Ala 260 265 270

Leu Arg Gln Lys Asn Val His Glu Val Lys Asn His Lys Phe Thr Ala 275 280 285

Arg Phe Phe Lys Gln Pro Thr Phe Cys Ser His Cys Thr Asp Phe Ile 290 295 300

Trp Gly Phe Gly Lys Gln Gly Phe Gln Cys Gln Val Cys Cys Phe Val 305 310 315 320

Val His Lys Arg Cys His Glu Phe Val Thr Phe Ser Cys Pro Gly Ala 325 330 335

Asp Lys Gly Pro Ala Ser Asp Asp Pro Arg Ser Lys His Lys Phe Lys 340 345 350

Ile His Thr Tyr Ser Ser Pro Thr Phe Cys Asp His Cys Gly Ser Leu 355 360 365

Leu Tyr Gly Leu Ile His Gln Gly Met Lys Cys Asp Thr Cys Met Met 370 375 380

Asn Val His Lys Arg Cys Val Met Asn Val Pro Ser Leu Cys Gly Thr 385 390 395 400

Asp His Thr Glu Arg Arg Gly Arg Ile Tyr Ile Gln Ala His Ile Asp
405 410 415

Arg Asp Val Leu Ile Val Leu Val Arg Asp Ala Lys Asn Leu Val Pro 420 425 430

Met Asp Pro Asn Gly Leu Ser Asp Pro Tyr Val Lys Leu Lys Leu Ile 435 440 445

Pro Asp Pro Lys Ser Glu Ser Lys Gln Lys Thr Lys Thr Ile Lys Cys 450 455 460

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His Ala Val Phe Tyr Ala Ala Glu Ile Ala Ile Gly Leu Phe Phe Leu 690 695 700

Gln Ser Lys Gly Ile Ile Tyr Arg Asp Leu Lys Leu Asp Asn Val Met 705 710 715 720

Leu Asp Ser Glu Gly His Ile Lys Ile Ala Asp Phe Gly Met Cys Lys
725 730 735

Glu Asn Ile Trp Asp Gly Val Thr Thr Lys Thr Phe Cys Gly Thr Pro $740 \hspace{1.5cm} 745 \hspace{1.5cm} 750$

Asp Tyr Ile Ala Pro Glu Ile Ile Ala Tyr Gln Pro Tyr Gly Lys Ser 755 760 765

Val Asp Trp Trp Ala Phe Gly Val Leu Leu Tyr Glu Met Leu Ala Gly 770 780

Gln Ala Pro Phe Glu Gly Glu Asp Glu Asp Glu Leu Phe Gln Ser Ile 785 790 795 800

Met Glu His Asn Val Ala Tyr Pro Lys Ser Met Ser Lys Glu Ala Val 805 810 815

Ala Ile Cys Lys Gly Leu Met Thr Lys His Pro Gly Lys Arg Leu Gly 820 825 830

Cys Gly Pro Glu Gly Glu Arg Asp Ile Lys Glu His Ala Phe Phe Arg 835 840 845

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Lys Pro Lys Ala Arg Asp Lys Arg Asp Thr Ser Asn Phe Asp Lys Glu 865 870 880

Phe Thr Arg Gln Pro Val Glu Leu Thr Pro Thr Asp Lys Leu Phe Ile 885 890 895

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Asp Pro Ser Gln Asn Thr Ala Gln Leu Asp Gln Phe Asp Arg Ile Lys

35 40 45

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_			His								_				_	152
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145	1110	+ y +	nia	ALG	150	110	Vai	ьса	1111	155		ıyı	пеп	1113	160	
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нец	дар	rea	TIE	165	Arg	ASP	ьец	пуз	170	GIU	ASII	ъеп	ьeu		Asp	
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GTII	GIII	GIY			GIN	vaı	THE			GTĀ	Pne	Ala	_	_	Val	
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Lys	GLy			Trp	Thr	Leu			Thr	Pro	Glu			Ala	Pro	
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										Lys		_	_			010
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Ту	: Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Pro	Lys	
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	ggc			-										_	3 3	1584
Ası	ı Gly			Val	Asn	Phe			Arg	His	Asn	ılle	Lys	Asp	Gly	
		515	٠				520					525	ı			
	gtt															1632
Se	r Val		Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	: Gl	/ Asp	
	530)				535					540)				

Gly P					Pro					Leu		_	caa Gln		-	1680
545					550					555					560	
ctt t															-	1728
Leu S	er	Lys	Asp	Pro 565	Asn	Glu	Lys	Arg	Asp 570	His	Met	Ile	Leu	Leu 575	Glu	
ttt g																1776
Phe V	al	Thr		Ala	Gly	Ile	Thr		Gly	Met	Asp	Glu	Leu	Tyr	Lys	
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cct c	-		taa													1788
Pro G	ln		*													
		595														
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	<2	11>	595													
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	<4	00>	2													
Met G				Ala	Ala	Ala	Lys	Lys	Gly	Ser	Glu	Gln	Glu	Ser	Val	
Met G				Ala 5	Ala	Ala	Lys	Lys	Gly 10	Ser	Glu	Gln	Glu	Ser 15	Val	
	lу	Asn	Ala	5					10					15		
1 Lys G	Sly Slu	Asn Phe	Ala Leu 20	5 Ala	Lys	Ala	Lys	Glu 25	10 Asp	Phe	Leu	Lys	Lys 30	15 Trp	Glu	
1	Sly Slu	Asn Phe Ser	Ala Leu 20	5 Ala	Lys	Ala	Lys Gln	Glu 25	10 Asp	Phe	Leu	Lys	Lys 30	15 Trp	Glu	
1 Lys G Asp P	Slu Slu Pro	Asn Phe Ser 35	Ala Leu 20 Gln	5 Ala Asn	Lys Thr	Ala Ala	Lys Gln 40	Glu 25 Leu	10 Asp Asp	Phe Gln	Leu Phe	Lys Asp 45	Lys 30 Arg	15 Trp Ile	Glu Lys	
1 Lys G Asp P	Slu Slu Pro	Asn Phe Ser 35	Ala Leu 20 Gln	5 Ala Asn	Lys Thr	Ala Ala Phe	Lys Gln 40	Glu 25 Leu	10 Asp Asp	Phe Gln	Leu Phe Leu	Lys Asp 45	Lys 30 Arg	15 Trp Ile	Glu Lys	
1 Lys G Asp P Thr L	Slu Pro Leu	Asn Phe Ser 35 Gly	Ala Leu 20 Gln Thr	5 Ala Asn Gly	Lys Thr Ser	Ala Ala Phe 55	Lys Gln 40 Gly	Glu 25 Leu Arg	10 Asp Asp Val	Phe Gln Met	Leu Phe Leu 60	Lys Asp 45 Val	Lys 30 Arg Lys	15 Trp Ile	Glu Lys Lys	
Lys G Asp P Thr L 5 Glu S	Slu Pro Leu	Asn Phe Ser 35 Gly	Ala Leu 20 Gln Thr	5 Ala Asn Gly	Lys Thr Ser	Ala Ala Phe 55	Lys Gln 40 Gly	Glu 25 Leu Arg	10 Asp Asp Val	Phe Gln Met	Leu Phe Leu 60	Lys Asp 45 Val	Lys 30 Arg Lys	15 Trp Ile	Glu Lys Lys Val	
Lys G Asp P Thr L 5 Glu S 65	Gly Glu Pro Leu GO	Asn Phe Ser 35 Gly	Ala Leu 20 Gln Thr	5 Ala Asn Gly	Lys Thr Ser Tyr 70	Ala Ala Phe 55 Ala	Lys Gln 40 Gly Met	Glu 25 Leu Arg	10 Asp Asp Val Ile	Phe Gln Met Leu 75	Leu Phe Leu 60 Asp	Lys Asp 45 Val	Lys 30 Arg Lys	15 Trp Ile His	Glu Lys Lys Val 80	
Lys G Asp P Thr L 5 Glu S	Gly Glu Pro Leu GO	Asn Phe Ser 35 Gly	Ala Leu 20 Gln Thr	5 Ala Asn Gly His	Lys Thr Ser Tyr 70	Ala Ala Phe 55 Ala	Lys Gln 40 Gly Met	Glu 25 Leu Arg	10 Asp Asp Val Ile	Phe Gln Met Leu 75	Leu Phe Leu 60 Asp	Lys Asp 45 Val	Lys 30 Arg Lys	15 Trp Ile His Lys	Glu Lys Lys Val 80	
Lys G Asp P Thr L 5 Glu S 65 Val L	Gly Glu Glu Geu Ger	Asn Phe Ser 35 Gly Gly Leu	Ala Leu 20 Gln Thr Asn	5 Ala Asn Gly His Gln 85	Lys Thr Ser Tyr 70 Ile	Ala Ala Phe 55 Ala Glu	Lys Gln 40 Gly Met	Glu 25 Leu Arg Lys	10 Asp Asp Val Ile Leu 90	Phe Gln Met Leu 75 Asn	Leu Phe Leu 60 Asp	Lys Asp 45 Val Lys	Lys 30 Arg Lys Gln Arg	15 Trp Ile His Lys Ile 95	Glu Lys Lys Val 80 Leu	
Lys G Asp P Thr L 5 Glu S 65	Gly Glu Glu Geu Ger	Asn Phe Ser 35 Gly Gly Leu	Ala Leu 20 Gln Thr Asn	5 Ala Asn Gly His Gln 85	Lys Thr Ser Tyr 70 Ile	Ala Ala Phe 55 Ala Glu	Lys Gln 40 Gly Met	Glu 25 Leu Arg Lys	10 Asp Asp Val Ile Leu 90	Phe Gln Met Leu 75 Asn	Leu Phe Leu 60 Asp	Lys Asp 45 Val Lys	Lys 30 Arg Lys Gln Arg	15 Trp Ile His Lys Ile 95	Glu Lys Lys Val 80 Leu	
Lys G Asp P Thr L 5 Glu S 65 Val L	Gly Glu Glu Go Ger Gy Mla	Asn Phe Ser 35 Gly Gly Leu Val	Ala Leu 20 Gln Thr Asn Lys Asn 100	5 Ala Asn Gly His Gln 85 Phe	Lys Thr Ser Tyr 70 Ile	Ala Ala Phe 55 Ala Glu Phe	Lys Gln 40 Gly Met His	Glu 25 Leu Arg Lys Thr	10 Asp Asp Val Ile Leu 90 Lys	Phe Gln Met Leu 75 Asn	Leu Phe Leu 60 Asp Glu Glu	Lys Asp 45 Val Lys Phe	Lys 30 Arg Lys Gln Arg Ser 110	15 Trp Ile His Lys Ile 95 Phe	Glu Lys Lys Val 80 Leu Lys	

Met	Phe	Ser	His	Leu	Arg	Arg	Ile	Gly	Arg	Phe	Ser	Glu	Pro	His	Ala
	130					135					140				
Arg	Phe	Tyr	Ala	Ala	Gln	Ile	Val	Leu	Thr	Phe	Glu	Tyr	Leu	His	Ser
145					150					155					160
Leu	Asp	Leu	Ile	Tyr	Arg	Asp	Leu	Lys	Pro	Glu	Asn	Leu	Leu	Ile	Asp
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Gln	Gln	Gly	Tyr	Ile	Gln	Val	Thr	Asp	Phe	Gly	Phe	Ala	Lys	Arg	Val
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Lys	Gly	Arg	Thr	Trp	Thr	Leu	Cys	Gly	Thr	Pro	Glu	Tyr	Leu	Ala	Pro
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Glu	Ile	Ile	Leu	Ser	Lys	Gly	Tyr	Asn	Lys	Ala	Val	Asp	Trp	Trp	Ala
	210					215					220				
Leu	Gly	Val	Leu	Ile	Tyr	Glu	Met	Ala	Ala	Gly	Tyr	Pro	Pro	Phe	Phe
225					230					235					240
Ala	Asp	Gln	Pro	Ile	Gln	Ile	Tyr	Glu	Lys	Ile	Val	Ser	Gly	Lys	Val
				245					250					255	
Arg	Phe	Pro	Ser	His	Phe	Ser	Ser	Asp	Leu	Lys	Asp	Leu	Leu	Arg	Asn
			260					265					270		
Leu	Leu	Gln	Val	Asp	Leu	Thr	Lys	Arg	Phe	Gly	Asn	Leu	Lys	Asp	Gly
		275					280					285			
Val		Asp	Ile	Lys	Asn	His	Lys	Trp	Phe	Ala	Thr	Thr	Asp	Trp	Ile
	290					295					300				
	Ile	Tyr	Gln	Arg		Val	Glu	Ala	Pro	Phe	Ile	Pro	Lys	Phe	Lys
305	_				310					315					320
GLY	Pro	Gly	Asp		Ser	Asn	Phe	Asp	Asp	Tyr	Glu	Glu	Glu		Ile
.	••	_		325					330					335	
Arg	vaı	Ser		Asn	Glu	Lys	Cys		Lys	Glu	Phe	Thr		Phe	Gly
70	70.7		340	_				345					350		
Arg	Ата		Ser	Lys	GIY	GLu		Leu	Phe	Thr	Gly		Val	Pro	Ile
T 0.11	17_]	355	-	_	~ 3	-	360	_			_	365			
Leu	370	GIU	Leu	Asp	ета		vaı	Asn	Gly	Gln		Phe	Ser	Val	Ser
C1.,		C1	C1	G1	3 3. –	375	FT.3		~ 1	_	380		_	_	
385	GIU	GTÀ	GIU	GTÀ		Ата	Thr	Tyr	Gly		Leu	Thr	Leu	Lys	
	C	m h	m)	G3	390	.			_	395			_		400
116	Cys	Inr	Thr		ьуѕ	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu		Thr
Thγ	T 0	ШЪ	m	405	773	61	_	5 1	410	_	_	_	_	415	
TIIT	neu	THE		етй	vai	GIN	cys		Ser	Arg	туr	Pro		His	Met
Luc	G1~	u: ~	420	DF -	Dh -	T •	C- :	425	N# = 1	_	C.3	63	430		
пÃр	GTII	435	Asp	rne	rne	гÀг		Ala	Met	Pro	Glu		Tyr	Val	Glr
		300					440					445			

Glu	Arg 450	Thr	Ile	Phe	Tyr	Lys 455	Asp	Asp	Gly	Asn	Tyr 460	Lys	Thr	Arg	Ala		
Glu		Lys	Phe	Glu	Gly		Thr	Leu	Val	Asn		Ile	Glu	Leu	Lys		
465					470					475					480		
Gly	Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Met	Glu		
				485					490		_		_	495			
Tyr	Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Pro	Lys		
			500					505					510				
Asn	Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Lys	Asp	Gly		
		515					520					525					
Ser	Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp		
	530					535					540						
Gly	Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala		
545					550					555					560		
Leu	Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Ile	Leu	Leu	Glu		
				565					570					575			
Phe	Val	Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Lys		
			580					585					590				
Pro	Gln	Glu															
		595															
		210>															
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			DNA				,	,									
	<.	213>	Aeq	uore	a vı	ctor	ıa a	na m	ouse								
	_	220>															
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													_	_	Val		
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gcc	aac	cgc	ttc	gcc	cgc	aaa	ggg	gcg	ctg	agg	caq	aaq	aac	gtg	cat	9	6
															His		
			20					25		_		-	30				
gag	gtg	aaa	gac	cac	aaa	tto	ato	gcc	: cgc	ttc	ttc	: aag	саа	ccc	acc	14	4

Glu	Val	Lys 35	Asp	His	Lys	Phe	Ile 40	Ala	Arg	Phe	Phe	Lys 45	Gln	Pro	Thr		
							10				•	15					
ttc	tgc	agc	cac	tgc	acc	gac	ttc	atc	tgg	ggg	ttt	ggg	aaa	caa	ggc	19	92
Phe	_	Ser	His	Cys	Thr	_	Phe	Ile	Trp	Gly		Gly	Lys	Gln	Gly		
	50					55					60						
ttc	cag	tgc	caa	gtt	tgc	tgt	ttt	gtg	gtt	cat	aag	agg	tgc	cat	gag	2	40
Phe	Gln	Cys	Gln	Val	Cys	Cys	Phe	Val	Val	His	Lys	Arg	Cys	His	Glu		
65					70					75					80		
ttc	gtt	acg	ttc	tct	tgt	ccg	ggt	gcg	gat	aag	gga	cct	gac	act	gac	2	88
Phe	Val	Thr	Phe	Ser	Cys	Pro	Gly	Ala	Asp	Lys	Gly	Pro	Asp	Thr	Asp		
				85					90					95			
gac	ccc	agg	agc	aag	cac	aag	ttc	aaa	atc	cac	aca	tac	gga	agc	cct	3	36
Asp	Pro	Arg	Ser	Lys	His	Lys	Phe	Lys	Ile	His	Thr	Tyr	Gly	Ser	Pro		
			100					105					110				
acc	ttc	tgt	gat	cac	tgt	ggg	tcc	ctg	ctc	tat	gga	ctt	atc	cac	caa	3	84
Thr	Phe	Cys	Asp	His	Cys	Gly	Ser	Leu	Leu	Tyr	Gly	Leu	Ile	His	Gln		
		115					120					125					
ggg	atg	aaa	t gt	gac	acc	tgc	gac	atg	aat	gtt	cac	aac	cag	tgt	gtg	4	32
Gly	Met	Lys	Cys	Asp	Thr	Cys	Asp	Met	Asn	Val	His	Asn	Gln	Cys	Val		
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Ile	Asn	Asp	Pro	Ser	Leu	Cys	Gly	Met	Asp	His	Thr	Glu	Lys	Arg	Gly		
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Arg	Ile	Tyr	Leu	Lys	Ala	Glu	Val	Thr	Asp	Glu	Lys	Leu	His	Val	Thr		
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gta	. cga	gat	gca	aaa	aat	cta	atc	cct	atg	gat	cca	aat	ggg	ctt	tcg	ţ	576
Val	Arg	Asp	Ala	Lys	Asn	Leu	Ile	Pro	Met	Asp	Pro	Asn	Gly	Leu	Ser		
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Asp	Pro	Tyr 195	Val	Lys	Leu		Leu 200	Ile	Pro	Asp	Pro	Lys 205	Asn	Glu	Ser		
aaa	cag	aaa	acc	aaa	acc	atc	cgc	tcc	aac	ctg	aat	cct	cag	tgg	aat	(672
Lys	Gln	Lys	Thr	Lys	Thr	Ile	Arg	Ser	Asn	Leu	Asn	Pro	Gln	Trp	Asn		
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Glu	Ser	Phe	Thr	Phe	Lys	Leu	Lys	Pro	Ser	Asp	Lys	Asp	Arg	Arg	Leu		
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Ser	Val	Glu	Ile	Trp	Asp	Trp	Asp	Arg	Thr	Thr	Arg	Asn	Asp	Phe	Met		
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Gly	Ser	Leu	Ser	Phe	Gly	Val	Ser	Glu	Leu	Met	Lys	Met	Pro	Ala	Ser		
			260					265					270				
gga	tgg	tat	aaa	gct	cac	aac	caa	gaa	gag	ggc	gaa	tat	tac	aac	gtg		864
Gly	Trp	Tyr 275	Lys	Ala	His	Asn	Gln 280	Glu	Glu	Gly	Glu	Tyr 285	Tyr	Asn	Val		
ccc	att	cca	gaa	qqa	gat	gaa	qaa	ggc	aac	atq	gaa	ctc	agg	cag	aag		912
Pro	Ile	Pro	Glu	Gly	Asp	Glu	Glu	Gly	Asn	Met	Glu	Leu	Arg	Gln	Lys		
	290					295					300	•					
ttt	gag	aaa	gcc	aag	cta	ggt	cct	gtt	ggt	aac	aaa	gto	ato	ago	cct		960
Phe	Glu	Lys	Ala	Lys	Leu	Gly	Pro	Val	Gly	Asn	Lys	: Val	Ile	Ser	Pro		
305					310					315					320		
tca	gaa	gac	: aga	aag	caa	cca	tcc	aac	aac	ctg	gac	aga	gtg	aaa	ctc	-	1008
Ser	Glu	Asp	Arg	Lys	Gln	Pro	Ser	Asn	Asn	Leu	Asp	Arg	, Val	Lys	Leu		
				325	ı				330	•				335	,		
aca	gac	: ttc	aac	: ttc	ctc	atg	gtg	ctg	ggg	aag	ggg	g agt	ttt	ggg	g aag	:	1056
Thr	Asp	Phe	e Asn	Phe	Leu	Met	Val	Leu	Gly	. Lys	Gly	y Ser	Phe	Gly	/ Lys		
			340	•				345	S				350)			
gtg	atç	; ctt	gct	gac	agg	aag	gga	acc	gag	g gaa	a cto	g tac	gco	ato	aag		1104

Val Met Leu 355	Ala Asp <i>i</i>	-	Sly Thr 360	Glu Glu	Leu Tyr 365	Ala Ile	Lys	
atc ctg aag	aag gac	gtg gtg a	atc cag	gac gac	gac gtg	gag tgc	acc 1152	
Ile Leu Lys	Lys Asp	Val Val I	Ile Gln	Asp Asp	Asp Val	Glu Cys	Thr	
370		375			380			
atg gtg gag			_				_	
Met Val Glu			Ala Leu	_	Lys Pro	Pro Phe		
385		390		395			400	
aca cag ctg	cac tcc	tgc ttc c	cag aca	gtg gac	cgg ctg	tac tto	gtc 1248	
Thr Gln Leu		Cys Phe (Gln Thr	_	Arg Leu	-		
	405			410		415		
atg gaa tac	gtc aac	ggc ggg g	gat ctt	atg tac	cac att	cag caa	gtc 1296	
Met Glu Tyr		Gly Gly A	-	Met Tyr	His Ile		Val	
	420		425			430		
ggg aaa ttt	aag gag	cca caa	gca gta	ttc tac	gca gcc	gag ato	e tcc 1344	
Gly Lys Phe	Lys Glu			Phe Tyr			e Ser	
435			440		445			
atc gga ctg			_				_	
Ile Gly Leu	Phe Phe		Lys Arg	Gly Ile		Arg As	Leu	
450		455			460			
aag ctg aac	-	•					-	
Lys Leu Asn	Asn Val		Asn Ser	-	His Ile	Lys Il		
465		470		475			480	
gac ttc ggg	-					_	-	
Asp Phe Gly	_	Lys Glu	His Met	_	Gly Val		_	
	485			490		49	5	
acc ttc tgc						=		
Thr Phe Cys	_	Pro Asp	_		Glu Ile		a Tyr	
	500		505			510		
cag ccg tac	ggg aag	tct gta	gat t gg	tgg gcg	tac gg	t gtg ct	g ctg 1584	

Gln	Pro	Tyr	Gly	Lys	Ser	Val	Asp	Trp	Trp	Ala	Tyr	Gly	Val	Leu	Leu	
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											•					
								ccg								1632
Tyr	530	Met	Leu	Ата	GTA		Pro	Pro	Phe	Asp	-	Glu	Asp	Giu	Asp	
	330					535					540					
gaa	ctg	ttt	cag	tct	ata	atg	gag	cac	aac	gtg	tcc	tac	ccc	aaa	tcc	1680
Glu	Leu	Phe	Gln	Ser	Ile	Met	Glu	His	Asn	Val	Ser	Tyr	Pro	Lys	Ser	
545					550					555					560	
ttg	tcc	aag	gaa	gcc	gtc	tcc	atc	tgc	aaa	gga	ctt	atg	acc	aaa	cag	1728
Leu	Ser	Lys	Glu		Val	Ser	Ile	Cys	Lys	Gly	Leu	Met	Thr	Lys	Gln	
				565					570					575		
cct	900	224	222	at a	a ~0	+ ~ ~	~~~	000	~~~	~~~	~~~	. ~ ~	~~+	~ + ~		1776
		_	_	_		_		ccc Pro					-	_		1776
110		כעם	580	Dea	Gry	Cys	Ory	585	014	Gry	O.L.U.	n. g	590	Val	nig	
													030			
gag	cat	gcc	ttc	ttc	agg	agg	atc	gac	tgg	gag	aaa	ctg	gag	aac	agg	1824
Glu	His	Ala	Phe	Phe	Arg	Arg	Ile	Asp	Trp	Glu	Lys	Leu	Glu	Asn	Arg	
		595					600					605				
						_		aaa -		-				-	-	1872
GIU	610		Pro	Pro	Phe		Pro	Lys	Val	Cys	_	_	Gly	Ala	Glu	
	010					615					620					
aac	ttt	gac	aag	ttc	ttc	acg	cga	gga	cag	cct	gto	tta	aca	cca	cca	1920
								Gly								
625					630					635					640	٠
gat	cag	ctg	gtc	att	gct	aac	ata	gac	caa	tct	gat	ttt	gaa	ggg	ttc	1968
Asp	Gln	Leu	Val	Ile	Ala	Asn	Ile	Asp	Gln	Ser	Asp	Phe	Glu	Gly	Phe	
				645					650					655	•	
t.c.~	+-+	~+ -				+++	~+	~	6 5-	_+ -		. == .	. a	· ~	~=-	2016
								cac His							gta Val	2016
	- À T	val	660		GTH	FIIG	val	665		, 116	. net	ı Gil	670		val	
													3,0	•		
ggg	cgc	gcc	atg	agt	aaa	gga	gaa	gaa	ctt	tto	: act	gga	gtt	gtc	: cca	2064

Gly	Arg	Ala 675	Met	Ser	Lys	Gly	Glu 680	Glu	Leu	Phe	Thr	Gly 685	Val	Val	Pro	
att	ctt	gtt	gaa	tta	gat	ggc	gat	gtt	aat	ggg	caa	aaa	ttc	tct	gtt	2112
Ile	Leu	Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	Gln	Lys	Phe	Ser	Val	
	690					695					700					
agt	gga	gag	ggt	gaa	ggt	gat	gca	aca	tac	gga	aaa	ctt	acc	ctt	aaa	2160
Ser	Gly	Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	
705					710					715					720	
ttt	att	tgc	act	act	ggg	aag	cta	cct	gtt	сса	tgg	cca	acg	ctt	gtc	2208
Phe	Ile	Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	
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act	act	ctc	act	tat	ggt	gtt	caa	tgc	ttt	tct	aga	tac	сса	gat	cat	2256
Thr	Thr	Leu		Tyr	Gly	Val	Gln	_	Phe	Ser	Arg	Tyr		Asp	His	
			740					745					750			
		-		-											gta	2304
Met	Lys			Asp	Phe	Phe	-		Ala	Met	Pro		_	Tyr	Val	
		755					760					765				
		_													cgt	2352
Gln		_	Thr	Ile	Phe			Asp	Asp	Gly			Lys	Thr	Arg	
	770					775					780					
			_												tta -	2400
		ı Val	. Lys	Phe			Asp	Thr	Leu			n Arg	l TT∈	e GII	Leu	
785					790					795	•				800	
			_			_	_								a atg	2448
Lys	Gly	, Il€	e Asp			Glu	Asp	Gl3			e Leu	ı Gly	y His		s Met	
				805	•				810)				81	ō	
gaa	tac	: aat	: tat	aac	: tca	cat	: aat	gta	a tao	c ato	c ato	g gc	a gad	c aaa	a cca	2496
Glu	туз	: Ası	-		n Ser	His	: Ası		_	r Ile	e Met	t Ala		_	s Pro	
,			820)				825	5				830	0		
aag	y aat	gg	c ato	c aaa	a gtt	aac	tto	c aaa	a at	t ag	a ca	c aa	c at	t aa	a gat	2544

Lys	Asn	Gly 835	Ile	Lys	Val	Asn	Phe 840	Lys	Ile	Arg	His	Asn 845	Ile	Lys	Asp	
gga	agc	gtt	caa	tta	gca	gac	cat	tat	caa	caa	aat	act	cca	att	ggc	2592
Gly	Ser	Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	
	850					855					860					
gat	ggc	cct	gtc	ctt	tta	cca	gac	aac	cat	tac	ctg	tcc	acg	caa	tct	2640
Asp	Gly	Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	
865					870					875					880	
gcc	ctt	tcc	aaa	gat	ccc	aac	gaa	aag	aga	gat	cac	atg	atc	ctt	ctt	2688
Ala	Leu	Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Ile	Leu	Leu	
				885					890					895		
gag	ttt	gta	aca	gct	gct	ggg	att	aca	cat	ggc	atg	gat	gaa	cta	tac	2736
Glu	Phe	Val	Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	
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aaa	cct	cag	gag	taa												2751
Lys	Pro	Gln	Glu	*												
		915														
	<	210>	4													
	<	211>	916													
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Met 1	Ala	Asp	Val	Tyr 5	Pro	Ala	Asn	Asp	Ser 10	Thr	Ala	Ser	Gln -	Asp 15	Val	
Ala	Asn	Arg	Phe	Ala	Arg	Lys	Gly	Ala	Leu	Arg	Gln	Lys	Asn	Val	His	
			20					25					30			
Glu	Val	Lys	Asp	His	Lys	Phe	Ile	Ala	Arg	Phe	Phe	: Lys	Gln	Pro	Thr	
		35					40					45				
Phe	Cys 50	Ser	His	Cys	Thr	Asp 55	Phe	lle	Trp	Gly	Phe	e Gly	Lys	s Glr	n Gly	
Phe	Gln	Cys	Gln	Val	Cys	Cys	Phe	. Val	Val	His	Lys	s Arg	Суз	His	s Glu	
65					70					75					80	

Phe	Val	Thr	Phe	Ser 85	Cys	Pro	Gly	Ala	Asp 90	Lys	Gly	Pro	Asp	Thr 95	Asp
Asp	Pro	Arg	Ser 100	Lys	His	Lys	Phe	Lys 105	Ile	His	Thr	Tyr	Gly 110	Ser	Pro
Thr	Phe	Cys 115	Asp	His	Cys	Gly	Ser 120	Leu	Leu	Tyr	Gly	Leu 1 25	Ile	His	Gln
Gly	Met 130	Lys	Cys	Asp	Thr	Cys 135	Asp	Met	Asn	Val	His	Asn	Gln	Cys	Val
Ile	Asn	Asp	Pro	Ser	Leu	Cys	Gly	Met	Asp	His	Thr	Glu	Lys	Arg	Gly
145					150					155					160
Arg	Ile	Tyr	Leu	Lys	Ala	Glu	Val	Thr	Asp	Glu	Lys	Leu	His	Val	Thr
				165					170					175	
Val	Arg	Asp		Lys	Asn	Leu	Ile		Met	Asp	Pro	Asn	-	Leu	Ser
7	D	m	180	.	.	.	7	185	D	3	5	-	190	61	_
Asp	Pro	Tyr 195	vaı	ьуѕ	Leu	гла	ьеи 200	ire	Pro	Asp	Pro	Lys 205	Asn	Glu	Ser
Lys	Gln		Thr	Lys	Thr	Ile		Ser	Asn	Leu	Asn		Gln	Trp	Asn
-	210	-		4		215					220			1	
Glu	Ser	Phe	Thr	Phe	Lys	Leu	Lys	Pro	Ser	Asp	Lys	Asp	Arg	Arg	Leu
225					230					235					240
Ser	Val	Glu	Ile	Trp	Asp	Trp	Asp	Arg	Thr	Thr	Arg	Asn	Asp	Phe	Met
				245					250					255	
Gly	Ser	Leu	Ser	Phe	Gly	Val	Ser	Glu	Leu	Met	Lys	Met	Pro	Ala	Ser
			260					265					270		
Gly	Trp	Tyr	Lys	Ala	His	Asn	Gln	Glu	Glu	Gly	Glu	Tyr	Tyr	Asn	Val
		275					280					285			
Pro		Pro	Glu	Gly	Asp		Glu	Gly	Asn	Met		Leu	Arg	Gln	Lys
Dl	290	-		_	_	295	~	** 3	61	_	300			_	_
	GLu	гàг	Ala	Lys			Pro	Vai	GLY			Val	Ile	Ser	Pro
305	Cl.	λon	7/~~	T ***	310		cor	7) on	7 an	315		71 ~~ ~	77-1	τ	320
261	GIU	Asp	Arg	ъуs 325	GTII	PIO	ser	ASII	330	Leu	ASP	Arg	vaı	335	
Thr	Asp	Phe	Asn		Leu	Met	Val	Lėu		Lys	Glv	Ser	Phe		
	_		340					345	-	-	-		350		-
Val	Met	Leu	Ala	Asp	Arg	Lys	Gly	Thr	Glu	Glu	Leu	Tyr	Ala	Ile	Lys
		355					360					365			
Ile	Leu	Lys	Lys	Asp	Val	Val	Ile	Gln	Asp	Asp	Asp	Val	Glu	Cys	Thr
	370					375					380				
Met	Val	Glu	Lys	Arg	Val	Leu	Ala	Leu	Leu	Asp	Lys	Pro	Pro	Phe	Leu
385					390					395	•				400

WO 00/23615

PCT/DK99/00562

Thr	Gln	Leu	His	Ser	Cys	Phe	Gln	Thr	Val	Asp	Arg	Leu	Tyr	Phe	Val
				405					410					415	
Met	Glu	Tyr	Val	Asn	Gly	Gly	Asp	Leu	Met	Tyr	His	Ile	Gln	Gln	Val
			420					425					430		
Gly	Lys	Phe	Lys	Glu	Pro	Gln	Ala	Val	Phe	Tyr	Ala	Ala	Glu	Ile	Ser
		435					440					445			
Ile	Gly	Leu	Phe	Phe	Leu	His	Lys	Arg	Gly	Ile	Ile	Tyr	Arg	Asp	Leu
	450					455					460				
Lys	Leu	Asn	Asn	Val	Met	Leu	Asn	Ser	Glu	Gly	His	Ile	Lys	Ile	Ala
465					470					475					480
Asp	Phe	Gly	Met	Cys	Lys	Glu	His	Met	Met	Asp	Gly	Val	Thr	Thr	Arg
				485					490					495	
Thr	Phe	Cys	Gly	Thr	Pro	Asp	Tyr	Ile	Ala	Pro	Glu	Ile	Ile	Ala	Tyr
			500					505					510		
Gln	Pro	Tyr	Gly	Lys	Ser	Val	Asp	Trp	Trp	Ala	Tyr	Gly	Val	Leu	Leu
		515					520					525			
Tyr	Glu	Met	Leu	Ala	Gly	Gln	Pro	Pro	Phe	Asp	Gly	Glu	Asp	Glu	Asp
	530					535					540				
Glu	Leu	Phe	Gln	Ser	Ile	Met	Glu	His	Asn	Val	Ser	Tyr	Pro	Lys	Ser
545					550					555					560
Leu	Ser	Lys	Glu	Ala	Val	Ser	Ile	Суз	Lys	Gly	Leu	Met	Thr	Lys	Gln
				565					570					575	
Pro	Ala	Lys	Arg	Leu	Gly	Cys	Gly	Pro	Glu	Gly	Glu	Arg	Asp	Val	Arg
			580					585					590		
Glu	His	Ala	Phe	Phe	Arg	Arg	Ile	Asp	Trp	Glu	Lys	Leu	Glu	Asn	Arg
		595					600					605			
Glu	Ile	Gln	Pro	Pro	Phe	Lys	Pro	Lys	Val	Cys	Gly	Lys	Gly	Ala	Glu
	610					615					620				
Asn	Phe	Asp	Lys	Phe	Phe	Thr	Arg	Gly	Gln	Pro	Val	Leu	Thr	Pro	Pro
625					630					635					640
Asp	Gln	Leu	ı Val	Ile	: Ala	Asn	Ile	Asp	Gln	Ser	Asp	Phe	Glu	Gly	Phe
				645	•				650	1			-	655	
Ser	Туг	Val	. Asn	Pro	Glr	Phe	· Val	His	Pro	Ile	Leu	Gln	Ser	Ala	Val
			660)				665	,				670)	
Gly	Arg	, Ala	Met	Ser	Lys	Gly	7 Glu	Glu	Lev	Phe	Thr	Gly	v Val	Val	Pro
		675	5				680	•				685	5		
Ile			Glu	ı Lev	ı Asp			Val	. Asr	ı Gly			Phe	Ser	Val
	690					695					700				
		/ Glu	1 Gl	/ Glu) Ala	Thr	Туг	_	_	Let	ı Thr	Let	Lys
705	•				710)				715	•				720

Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val 725 730 Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His 745 Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val 760 Gln Glu Arg Thr Ile Phe Tyr Lys Asp Asp Gly Asn Tyr Lys Thr Arg 775 Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arq Ile Glu Leu 790 Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Met 805 810 Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Pro 825 Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Lys Asp 840 Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly 855 Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser 870 875 Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Ile Leu Leu 885 890 Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr 905 910 Lys Pro Gln Glu 915 <210> 5 <211> 1896 <212> DNA <213> Aequorea victoria and human <220> <221> CDS <222> (1)...(1896) <400> 5 atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu 1 5 10

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Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
		35					40					45				
tac	200	200	aac	220	cta	CCC	ata	ccc	tgg	CCC	a.c.c	ctc	ata	200	3.00	192
-				_	-				Trp							152
СуЗ	50	1111	GTA	БУЗ	Deu	55	Val	110	rrp	110	60	Бец	vai	1111	1111	
	50					55					00					
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70					75					80	
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
				85					90					95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
																204
					_										ggc	384
vaı	ьуs			GIY	Asp	Thr			Asn	Arg	lle			гуѕ	Gly	
		115					120					125				
atc	gac	ttc	aad	gag	gac	aac	aac	ato	cta	aaa	cac	aad	cta	gad	tac	432
_															Tyr	
	130					135				_	140	-	•		-	
aac	tac	aac	ago	cac	aac	gto	tat	ato	: atg	gcc	gac	aag	cag	aag	, aac	480
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	s Asn	
145					150					155	•				160	
ggc	ato	aag	gtg	aac	: ttc	aag	ato	: cgc	cac	aac	: ato	gaç	gac	ggd	c agc	528
Gly	Ile	Lys	Val	. Asr	. Phe	Lys	Ile	e Arg	y His	Asn	ıle	e Glu	ı Asp	Gly	y Ser	
				165					170)				175	5	

• •	_		-	-							_	atc				576
Val	Gln	Leu	Ala 180	Asp	His	Tyr	Gln	Gln 185	Asn	Thr	Pro	Ile	Gly 190	Asp	Gly	
			100					103					150			
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctg	624
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	
		195					200					205				
agc	aaa	gac	ccc	aac	aaa	aaσ	cac	σat	cac	atα	atc	ctg	cta	gag	ttc	672
_		_				_	-	-		_	-	Leu	_			
	210	•				215	_				220					
		_	-									ctg				720
	Thr	Ala	Ala	Gly		Thr	Leu	Gly	Met		Glu	Leu	Tyr	Lys		
225					230					235					240	
gga	ctc	aga	tct	cga	qct	caa	gct	tcg	aat	tca	acc	atg	gcg	qcq	gcg	768
		_		_	_		-	_				Met				
_		_		245					250					255		
gcg	gct	cag	ggg	ggc	ggg	ggc	ggg	gag	ccc	cgt	aga	acc	gag	ggg	gtc	816
Ala	Ala	Gln	_	Gly	Gly	Gly	Gly			Arg	Arg	Thr		_	Val	
			260					265					270			
aac	cca	aaa	atc	cca	aaa	gag	ata	gag	ato	ata	aao	ı aaa	cad	cca	ttc	864
	_		_	_					_		_		_		Phe	
		275					280					285				
gac	gtg	ggc	ccg	cgc	: tac	acg	cag	ttg	cag	tac	ato	ggc	gag	ggc	gcg	912
Asp		_	Pro	Arg	Tyr			Lev	Gln	Tyr	Ile	e Gly	Glu	Gly	Ala	
	290)				295					300)				
tac	aac	: atc	ı atc	age	t too	racc	tat	: дас	: cac	: ata	cac	. aac	r act	. cac	gtg	960
	_	_	_	_											y Val	
305	_				310		_	_		315	-	-			320	
gcc	ato	aaq	g aag	ato	ago	ccc	: ttc	gaa	a cat	cag	g ac	c tad	tg(c caç	g cgc	1008
Ala	Ile	E Lys	s Lys	: Ile	e Ser	Pro) Phe	e Gli	ı His	s Glr	Th:	г Туз	c Cys	s Glr	n Arg	
				325	5				330)				335	5	

_					_		_	_	_		-			aat	-	1056
mr	rea	Arg	340	116	GIN	11e	Leu	345	Arg	rne	Arg	HIS	350	Asn	vaı	
atc	ggc	atc	cga	gac	att	ctg	cgg	gcg	tcc	acc	ctg	gaa	gcc	atg	aga	1104
Ile	Gly	11e 355	Arg	Asp	Ile	Leu	Arg 360	Ala	Ser	Thr	Leu	Glu 365	Ala	Met	Arg	
gat	gtc	tac	att	gtg	cag	gac	ctg	atg	gag	act	gac	ctg	tac	aag	ttg	1152
Asp	Val	Tyr	Ile	Val	Gln	Asp	Leu	Met	Glu	Thr	Asp	Leu	Tyr	Lys	Leu	
	370					375					380					
ctg	aaa	agc	cag	cag	ctg	agc	aat	gac	cat	atc	tġc	tac	ttc	ctc	tac	1200
	Lys	Ser	Gln	Gln	Leu	Ser	Asn	Asp	His	Ile	Cys	Tyr	Phe	Leu	Tyr	
385					390					395					400	
cag	atc	ctg	cgg	ggc	ctc	aag	tac	atc	cac	tcc	gcc	aac	gtg	ctc	cac	1248
Gln	Ile	Leu	Arg	Gly	Leu	Lys	Tyr	Ile	His	Ser	Ala	Asn	Val	Leu	His	
				405					410					415		
cga	gat	cta	aag	ccc	tcc	aac	ctg	ctc	agc	aac	acc	acc	tgc	gac	ctt	1296
Arg	Asp	Leu	Lys	Pro	Ser	Asn	Leu	Leu	Ser	Asn	Thr	Thr	Cys	Asp	Leu	
			420					425					430			
_		•	-			_	-			-	_		, ,		gac	1344
Lys	Ile	-	_	Phe	Gly	Leu		Arg	Ile	Ala	Asp	Pro	Glu	His	Asp	
		435					440					445				
				_						_	-				gcc	1392
His			Phe	Leu	Thr		Tyr	Val	Ala	Thr	_	_	Tyr	Arg	Ala	
	450					455					460	I	-			
cca	gag	atc	atg	ctg	aac	tcc	aag	ggc	tat	acc	aag	tcc	ato	gac	atc	1440
	Glu	Ile	Met	Leu	Asn	Ser	Lys	Gly	Tyr	Thr	Lys	Ser	Ile	Asp	Ile	
465					470					475					480	
tgg	tct	gtg	ggc	tgc	att	ctg	gct	gag	atg	cto	tct:	aac	: cgg	g ccc	atc	1488
Trp	Ser	Val	Gly	Cys	Ile	Leu	Ala	Glu	. Met	Leu	Ser	Asn	Arg	Pro	Ile	
				485	•				490)				495	5	

20

						ctg	_	_						,,		1536
Pne	Pro	GIŸ		Hls	Tyr	Leu	Asp		Leu	Asn	His	lle		GLY	lle	
			500					505					510			
ctq	ggc	tcc	сса	tcc	caq	gag	gac	ctq	aat	tat	atc	atc	aac	atq	aaq	1584
_					_	Glu	_	-		-				_	-	
	•	515					520			-		525			-	
gcc	cga	aac	tac	cta	cag	tct	ctg	ccc	tcc	aag	acc	aag	gtg	gct	tgg	1632
Ala	Arg	Asn	Tyr	Leu	Gln	Ser	Leu	Pro	Ser	Lys	Thr	Lys	Val	Ala	Trp	
	530					535					540					
					-	tca	-			-		-	-	_	-	1680
545	rys	Leu	Pne	Pro	ьуs 550	Ser	Asp	Ser	ьуѕ	555	Leu	Asp	Leu	Leu	_	
242					550					333					560	
cqq	atg	tta	acc	ttt	aac	ccc	aat	aaa	caa	atc	aca	ata	gag	σaa	aca	1728
	_					Pro								_		
				565					570					575		
ctg	gct	cac	ccc	tac	ctg	gag	cag	tac	tat	gac	ccg	acg	gat	gag	cca	1776
Leu	Ala	His	Pro	Tyr	Leu	Glu	Gln	Tyr	Tyr	Asp	Pro	Thr	Asp	Glu	Pro	
			580					585					590			
a+ a	~~~	~~~										-				1004
	-					acc Th~		-	_			_	_		Pro	1824
Val	AIG	595		FLO	rne	1117	600		Met	Giu	ъeп	605	_	ъеп	PIO	
												000				
aag	gag	cgg	ctg	aag	gag	ctc	atc	ttc	cag	gag	aca	gca	. cgc	tto	cag	1872
Lys	Glu	Arg	Leu	Lys	Glu	Leu	Ile	Phe	Gln	Glu	Thr	Ala	Arg	Phe	Gln	
	610					615					620	,	-			
				-	-	ccc										1896
		Val	Leu	Glu		Pro	*									
625					630											

<210> 6

<211> 631

<212> PRT

<213> Aequorea victoria and human

<400> 6

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Asp	Val	Gly	Pro	Arg	Tyr	Thr	Gln	Leu	Gln	Tyr	Ile	Gly	Glu	Gly	Ala
	290					295					300				
Tyr	Gly	Met	Val	Ser	Ser	Ala	Tyr	Asp	His	Val	Arg	Lys	Thr	Arg	Val
305					310					315					320
Ala	Ile	Lys	Lys	Ile	Ser	Pro	Phe	Glu	His	Gln	Thr	Tyr	Cys	Gln	Arg
				325					330					335	
Thr	Leu	Arg	Glu	Ile	Gln	Ile	Leu	Leu	Arg	Phe	Arg	His	Glu	Asn	Val
			340					345					350		
Ile	Gly	Ile	Arg	Asp	Ile	Leu	Arg	Ala	Ser	Thr	Leu	Glu	Ala	Met	Arg
		355					360					365			
Asp	Val	Tyr	Ile	Val	Gln	Asp	Leu	Met	Glu	Thr	Asp	Leu	Tyr	Lys	Leu
	370					375					380				
Leu	Lys	Ser	Gln	Gln	Leu	Ser	Asn	Asp	His	Ile	Суѕ	Tyr	Phe	Leu	Tyr
385					390					395					400
Gln	Ile	Leu	Arg	Gly	Leu	Lys	Tyr	Ile	His	Ser	Ala	Asn	Val	Leu	His
				405					410					415	
Arg	Asp	Leu	Lys	Pro	Ser	Asn	Leu			Asn	Thr	Thr	_	Asp	Leu
			420					425					430		
Lys	Ile	_	Asp	Phe	Gly	Leu	Ala	Arg	Ile	Ala	Asp			His	Asp
		435		_			440					445			
His		-	Phe	Leu	Thr		Tyr	Val	Ala	Thr	_	-	Tyr	Arg	Ala
	450			_	_	455		0 1	_		460			_	
		тте	Met	Leu		Ser	Lys	СТУ	Tyr			Ser	ile	Asp	
465		573	61	G	470	T	77.	C3	Mah	475		. 7	7	D	480
rrp	Ser	val	GTÀ	_		ьeu	Ala	GIU	мет 490		ser	ASII	Arg		
Pho	Dro	C1.		485		Ton	7.00	Cln			เมเล	T 1 0	Tou	495	
rne	PIO	вту	500		тАт	Leu	Asp	505		i ASI	nrs	TTE	510	_	, ire
Len	Gly	Sor			· Gln	Glu	Asp			Cue	. т1о	, Tle			Tue
neu	ч	515		. ser	GIII	. GIU	520		L DOI	г суз	, 116	525		ne c	. шуз
Δla	Δτο			. Len	Gl n	Ser	Leu		s Sar	~ T.376	· Thr			Δ 1 =	ነ ጥምኮ
nia	530		LIYL	neu	((31))	535			, 561	. Бус	540		- , , , ,	. Alc	1 111
Ala			. Pho	Pro	. T.vs		Asp	Ser	~ T.376	: 7\] :			. T.e.i	1 T.O.	ı Der
545		, псс	LIIC		550 550		. 1100	, 501	. Ly.	555		, ASE	, псс	a nec	560
		· T.e.ı	ነ ሞኮፖ	Phe			Asn	T.375	: Δτα			- Val	Gli	. Gli	
	,	. 200	• 1111	565			7 1101		570		, 1111	. , ,	- 010	575	
T _i ei:	1 Ala	Hic	: Pro			. Gla	ı Glr	ነ ጥኒን፣) Pro	n Thi	~ Asr		
		1	580	_			- 511	585	_		- * * \	- 4114	590		\
Val	. Ala	Glr			Ph∈	Thr	. Phe			t Gli	ı Lei	ı Asr			ı Pro
		595					600				,	60!			

23

Lys	Glu 610	Arg	Leu	Lys	Glu	Leu 615	Ile	Phe	Gln	Glu	Thr 620	Ala	Arg	Phe	Gln	
Pro	Gly	Val	T.en	Glu	Δla						-					
625	GLY	Val	пси	GIU	630	110										
020					000											
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	<2	220>														
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		100>														4.0
									acc							48
Met 1	vai	ser	гуѕ	GIY	GIU	GIU	ren	rne	Thr 10	стА	vai	vaı	Pro	11e	Leu	
Τ.				J					10					13		
atc	gag	cta	gac	aac	gac	qta	aac	aac	cac	aaq	ttc	agc	ata	tcc	aac	96
									His	_		_				
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
		35					40					45				
									tgg							192
cys	Thr 50		GTÀ	гÀг	ьeu	Pro 55	vai	Pro	Trp	Pro	Thr 60		Val	Thr	Thr	
	50					33					00	,				
cta	acc	tac	aac	ata	cao	tac	tto	agc	cac	tac	ccc	gac	cac	ato	aag	240
									Arq			-				
65		_	-		70	-			_	75		•			80	
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggo	: tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gl	y Tyr	Val	Gln	Glu	
				85					90					95	,	

cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag 336

Arg	Thr	Ile	Phe 100	Phe	Lys	Asp	Asp	Gly 105	Asn	Tyr	Lys	Thr	Arg 110	Ala	Glu		
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly		
		115					120					125					
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr		
	130					135					140						
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn		
145					150					155					160		
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528	
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser		
				165					170					175			
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576	
Val	Gln	Leu	Ala 180	Asp	His	Tyr	Gln	Gln 185	Asn	Thr	Pro	Ile	Gly 190	Asp	Gly		
ccc	gtg	ctg	ctg	ccc	gac	aac	çac	tac	ctg	agc	acc	caq	tcc	qcc	ctg	624	
									Leu			_		_	_		
		195					200					205					
agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672	
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe		
	210					215					220						
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag	tcc	720	
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Ser		
225					230					235					240		
gga	ctc	aga	tct	cga	act	caa	act	tca	aat	tca	acc	ato	t tca	tcc	atc	768	
									Asn							, 50	
		9		245					250			-300		255			
ttg	cca	ttc	acg	ccg	сса	gtt	gtg	aag	aga	ctg	ctg	gga	tgg	aag	aag	816	

Leu	Pro	Phe	Thr 260	Pro	Pro	Val	Val	Lys 265	Arg	Leu	Leu	Gly	Trp 270	Lys	Lys	
	_						_					_	aat Asn	5 5 5	-	864
													aag Lys			912
				_		_					_		acc Thr			960
	_				_	-				-		-	tct Ser	_		1008
		_	_						_	_		_	aca Thr 350	Thr		1056
		-	Phe		-						-		Arg		cag Gln	1104
		His	•			_			_			Cys	_		tgg Trp	1152
	Trp					Ser			_		Lys	•		_	aac Asn 400	1200
			_		. Asn			_		Glu	-	_	-		c cct n Pro	1248
tac	: cac	: tat	: caç	ı aga	ı gtt	gag	aca	сса	gtt	ttç	g cct	c cca	a gta	a tta	a gtg	1296

Tyr	His	Туг	Gln 420	Arg	Val	Glu	Thr	Pro 425	Val	Leu	Pro	Pro	Val 430	Leu	Val	
	_		Thr	gag Glu												1344
		s Sei		cca Pro												1392
	n Se:			att		-	-							-	_	1440
_		_		agt Ser 485	Asp		-	-		Gln	_		-			1488
		_	_	a cta 1 Leu)					Leu			_		His	_	1536
			u Gl:	g cca	-			Ser	-		-		Trp	_		1584
	_	а Ту		t gaa			Gln		_		-	ı Thr			_	1632
	r Gl	-		a cto		. Val	-				: Asp					1680
-	•	-	_		u Gly					n Val		-		_	e acg a Thr	1728
gt	a ga	a at	g ac	a ag	a ag	g cat	: ata	a gga	a ag	a gga	a gt	g cg	c tta	a ta	c tac	1776

27

Val	Glu	Met	Thr	Arg	Arg	His	Ile	Gly	Arg	Gly	Val	Arg	Leu	Tyr	Tyr
			580					585					590		

ata	ggt	ggg	gaa	gtt	ttt	gct	gag	tgc	cta	agt	gat	agt	gca	atc	ttt	1824
Ile	Gly	Gly	Glu	Val	Phe	Ala	Glu	Cys	Leu	Ser	Asp	Ser	Ala	Ile	Phe	
		595					600					605				

gtg	cag	agc	ccc	aat	tgt	aat	cag	aga	tat	ggc	tgg	cac	cct	gca	aca	1872
Val	Gln	Ser	Pro	Asn	Cys	Asn	Gln	Arg	Tyr	Gly	Trp	His	Pro	Ala	Thr	
	610					615					620					

gtg	tgt	aaa	att	cca	cca	ggc	tgt	aat	ctg	aag	atc	ttc	aac	aac	cag	1920
Val	Cys	Lys	Ile	Pro	Pro	Gly	Cys	Asn	Leu	Lys	Ile	Phe	Asn	Asn	Gln	
625					630					635					640	

gaa	ttt	gct	gct	ctt	ctg	gct	cag	tct	gtt	aat	cag	ggt	ttt	gaa	gcc	1968
Glu	Phe	Ala	Ala	Leu	Leu	Ala	Gln	Ser	Val	Asn	Gln	Gly	Phe	Glu	Ala	
				645					650					655		

gtc	tat	cag	cta	act	aga	atg	tgc	acc	ata	aga	atg	agt	ttt	gtg	aaa	2016
Val	Tyr	Gln	Leu	Thr	Arg	Met	Суз	Thr	Ile	Arg	Met	Ser	Phe	Val	Lys	
			660					665					670			

ggg	tgg	gga	gca	gaa	tac	cga	agg	cag	acg	gta	aca	agt	act	cct	tgc	2064
Gly	Trp	Gly	Ala	Glu	Tyr	Arg	Arg	Gln	Thr	Val	Thr	Ser	Thr	Pro	Cys	
		675					680					685				

tgg	att	gaa	ctt	cat	ctg	aat	gga	cct	cta	cag	tgg	ttg	gac	aaa	gta	2112
Trp	Ile	Glu	Leu	His	Leu	Asn	Gly	Pro	Leu	Gln	Trp	Leu	Asp	Lys	Val	
	690					695					700					

tta	act	cag	atg	gga	tcc	cct	tca	gtg	cgt	tgc	tca	agc	atg	tca	taa	2160
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Glu	Gly	Glu 35	Gly	Asp	Ala	Thr	Tyr 40	Gly	Lys	Leu	Thr	Leu 45	Lys	Phe	Ile
Cys	Thr 50	Thr	Gly	Lys	Leu	Pro 55	Val	Pro	Trp	Pro	Thr 60	Leu	Val	Thr	Thr
Leu 65	Thr	Tyr	Gly	Val	Gln 70	Cys	Phe	Ser	Arg	Tyr 75	Pro	Asp	His	Met	Lys 80
Gln	His	Asp	Phe	Phe 85	Lys	Ser	Ala	Met	Pro 90	Glu	Gly	Tyr	Val	Gln 95	Glu
Arg	Thr	Ile	Phe		Lys	Asp	Asp	Gly 105	Asn	Tyr	Lys	Thr	Arg 110	Ala	Glu
Val	Lys	Phe 115		Gly	Asp	Thr	Leu 120		Asn	Arg	Ile	Glu 125		Lys	Gly
Ile	Asp		e Lys	s Glu	Asp	Gly		Ile	Leu	Gly	His		Leu	Glu	Tyr
Asn	Туг	Ası	n Sei	His	s Asn	val	. Tyr	Ile	Met	: Ala	Asp	Lys	Gln	Lys	Asn
145					150					155					160
Gly	, Ile	e Ly	s Val	l Ası 165		e Lys	s Ile	e Arç	His		ı Ile	e Glu	ı Asp	Gly 175	Ser
Val	Glr	n Le	u Ala 18		o His	з Туз	c Glr	185		n Thi	: Pro	o Ile	e Gly 190		Gly
Pro	y Vai	l Le 19		u Pro	o Asp	o Ası	n His		: Lei	u Sei	r Thr	Gl:		Ala	a Leu
Sei	г Ly. 21		p Pr	o As	n Gl	ц L y	_	g Ası	o Hi	s Me	t Val		u Let	ı Gl	ı Phe
Va.		r Al	a Al	a Gl	y Il 23		r Le	u Gl	y Me	t As		ı Le	u Ty:	r Ly	s Ser 240
		u Ar	g Se	r Ar 24	g Al		n Al	a Se	r As 25	n Se		r Me	t Se	r Se 25	r Ile 5
Le	u Pr	o Ph	ne Th	ır Pr		o Va	l Va	1 Ly 26		g Le	u Le	u Gl	y Tr 27		s Lys
Se	r Al	a G1		y Se	er Gl	y G1	y Al 28		y Gl	.у Gl	y Gl	u Gl 28		n Gl	y Glr
Gl	u Gl	u Ly	ys Tı	cp C3	ys Gl	u Ly	s Al	a Va	ıl Ly	ıs Se	er Le	u Va	al Ly	s Ly	s Lei
	20	٠.				20	5				30	in			

Lys	ьуѕ	Thr	GTÄ	Arg	Leu	Asp	GIU	Leu	GIU	гÀг	АТА	тте	THE	IUL	GIII
305					310					315					320
Asn	Cys	Asn	Thr	Lys	Cys	Val	Thr	Ile	Pro	Ser	Thr	Cys	Ser	Glu	Ile
				325					330					335	
Trp	Gly	Leu	Ser	Thr	Pro	Asn	Thr	Ile	Asp	Gln	Trp	Asp	Thr	Thr	Gly
			340					345					350		
Leu	Tyr	Ser	Phe	Ser	Glu	Gln	Thr	Arg	Ser	Leu	Asp	Gly	Arg	Leu	Gln
		355					360					365			
Val	Ser	His	Arg	Lys	Gly	Leu	Pro	His	Val	Ile	Tyr	Cys	Arg	Leu	Trp
	370		_			375					380				
Arq	Trp	Pro	Asp	Leu	His	Ser	His	His	Glu	Leu	Lys	Ala	Ile	Glu	Asn
385	•		-		390					395	_				400
Cys	Glu	Tyr	Ala	Phe	Asn	Leu	Lys	Lys	Asp	Glu	Val	Cys	Val	Asn	Pro
-		-		405			_		410			_		415	
Tyr	His	Tyr	Gln	Arg	Val	Glu	Thr	Pro	Val	Leu	Pro	Pro	Val	Leu	Val
		_	420					425					430		
Pro	Arg	His	Thr	Glu	Ile	Leu	Thr	Glu	Leu	Pro	Pro	Leu	Asp	Asp	Tyr
		435					440					445			
Thr	His	Ser	Ile	Pro	Glu	Asn	Thr	Asn	Phe	Pro	Ala	Gly	Ile	Glu	Pro
	450					455					460				
Gln	Ser	Asn	Tyr	Ile	Pro	Glu	Thr	Pro	Pro	Pro	Gly	Tyr	Ile	Ser	Glu
465					470					475					480
Asp	Gly	Glu	Thr	Ser	Asp	Gln	Gln	Leu	Asn	Gln	Ser	Met	Asp	Thr	Gly
				485					490)				495	
Ser	Pro	Ala	Glu	Leu	Ser	Pro	Thr	Thr	Leu	Ser	Pro	Val	Asn	His	Ser
			500)				505	٠				510		
Leu	Asp	Leu	Gln	Pro	Val	Thr	Tyr	Ser	Glu	Pro	Ala	Phe	Trp	Cys	Ser
		515					520	ı				525	5		
Ile	Ala	Туг	Туг	Glu	ı Lev	a Asn	Gln	Arg	y Val	Gly	/ Gli	ı Thr	Phe	His	Ala
	530	•				535	.				540)			
Ser	Gln	Pro	Ser	Let	Thr	. Val	Asp	Gly	/ Phe	e Thi	: Asp	Pro	Ser	Asr	Ser
545					550)				555	5				560
Glu	Arg	J Ph∈	e Cys	s Lev	ı Gly	Lev	ı Lev	ser	: Asr	n Val	l Ası	n Ar	g Asr	n Ala	a Thr
				565	5				570)				575	5
Val	Glu	ı Met	Thi	c Arq	arç	g His	s Ile	e Gly	Arq	g Gl	y Val	l Ar	g Lei	туз	туг
			580)				585	5				590)	
Ile	: Gl	Gly	/ Glu	. Va	l Phe	e Ala	a Glu	і Суз	s Le	ı Se	r Ası	Se:	r Ala	a Ile	e Phe
		595	5				600)				60!	5		
Val	. Glr	ı Sei	r Pro	o Ası	а Суя	s Asr	n Glr	n Ar	g Ty:	r Gl	y Tr	o Hi	s Pro	o Ala	a Thi
	610)				615	5				62	0			

Val	Суѕ	Lys	Ile	Pro	Pro	Gly	Cys	Asn	Leu	Lys	Ile	Phe	Asn	Asn	Gln	
625					630					635					640	
Glu	Phe	Ala	Ala	Leu	Leu	Ala	Gln	Ser	Va1	Asn	Gln	Gly	Phe	Glu	Ala	
				645					650					655		
Val	Tyr	Gln	Leu	Thr	Arg	Met	Суѕ		Ile	Arg	Met	Ser		Val	Lys	
	_		660		_	_	_	665				_	670		_	
Gly	Trp	_	Ala	Glu	Tyr	Arg	_	GIn	Thr	Val	Thr		Thr	Pro	Cys	
M2000	T 7 -	675	T 0	n: a	T	7	680	Dro	Ton	C1 n	T	685	7	T	1703	
пр	690	GIU	Leu	nıs	ren	695	GIY	PIO	Leu	GIII	700	Leu	Asp	гда	Val	
Len		Gln	Met	Glv	Ser		Ser	Val	Ara	Cvs		Ser	Met	Ser		
705	1111	02	1300	01	710		001		9	715	501	001	1100	001		
	<:	210>	9													
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			CDS													
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	_	400>	a													
atq				tta	cca	ttc	acq	cca	г сса	att	ata	aad	aga	cta	ctg	48
_	_						_	_		-		_	-	_	Leu	
1				5					10			-	_	15		
gga	tgg	aag	aag	tca	gct	ggt	ggg	, tct	gga	gga	gca	ggc	gga	gga	gag	96
Gly	Trp	Lys	Lys	Ser	Ala	Gly	Gly	/ Ser	Gly	Gly	Ala	Gly	Gly	Gly	Glu	
			20					25	5				30)		
													-			
cag	aat	ggg	cag	gaa	gaa	aag	tgg	g tgt	gaç	aaa	gca	gto	, aaa	agt	: ctg	144
Gln	Asn	_		Glu	Glu	Lys			Glı	Lys	: Ala		_	s Sei	Leu	
		35	•				40)				45	5			
~+· -			. ــــ									المصي				102
										-					gcc Ala	192
A G 1	. Lys 50	_	nen	гъ	тλε	55		k wri	y nel	ı AS[6(GI		וגט ג	тъЛ;	o mid	
	J.C	•				<i>-</i>	•				01	•				
ato	acc	r act	caa	aac	t at	- aat	act	r aa:	a tai	- att	acc	r ata	a cc:	a ac	c act	240

Ile ' 65	Thr	Thr	Gln	Asn	Cys 70	Asn	Thr	Lys	Суѕ	Val 75	Thr	Ile	Pro	Ser		nr 80	
									cca								288
Cys	Ser	Glu	Ile	Trp 85	Gly	Leu	Ser	Thr	Pro 90	Asn	Thr	Ile	Asp	Glr 9!		rp	
									gaa								336
Asp	Thr	Thr	Gly 100		Tyr	Ser	Phe	Ser 105	Glu	Gln	Thr	Arg	110		u A	zsb	
									gga Gly								384
		115	1				120					125	•				
		Leu					Asp		cac ı His			s His					432
	att	: gaa							t aat								480
Ala 145		e Gli	ı Ası	n Cys	5 Glu 150		: Ala	a Phe	e Asr	тье: 15		з гу	S AS	р G.	Lu	160	
_	-								a gti g Val								528
-				16					17						75		
				l Pr					g at u Il 85				u Le				576
									ca ga								624
Le	u As	p As		r Tr	ır Hı	.s 5e	r 11 20		co Gl	u At) II I I)5	1			
	y Il	.e G				er As	n Ty				lu T					gga Gly	672
ta	21 t at		gt g	aa g	at g	21 ga ga		ca a	gt g	ac c			tg a	at	caa	ı agt	720

Tyr :	Ile	Ser	Glu	Asp	Gly 230	Glu	Thr	Ser	Asp	Gln 235	Gln	Leu	Asn	Gln	Ser 240	
atg	-															768
Met	Asp	Thr	Gly	Ser 245	Pro	Ala	Glu	Leu	Ser 250	Pro	Thr	Thr	Leu	Ser 255	Pro	
att	aat	cat	agc	tta	gat.	tta	caq	сса	att	act	tac	tca	gaa	cct	gca	816
												Ser				
			260					265					270			
ttt	tgg	tgt	tca	ata	gca	tat	tat	gaa	tta	aat	cag	agg	gtt	gga	gaa	864
Phe	Trp	Cys 275	Ser	Ile	Ala	Tyr	Tyr 280	Glu	Leu	Asn	Gln	Arg 285	Val	Gly	Glu	
acc	ttc	cat	gca	tca	cag	ccc	tca	ctc	act	gta	gat	ggc	ttt	aca	gac	912
Thr		His	Ala	Ser	Gln		Ser	Leu	Thr	Val	•	Gly	Phe	Thr	Asp	
	290					295					300					
cca	tca	aat	tca	gag	agg	ttc	tgc	tta	ggt	tta	ctc	tcc	aat	gtt	aac	960
	Ser	Asn	Ser	Glu	Arg	Phe	Суѕ	Leu	Gly	Leu	Leu	Ser	Asn	Val		
305					310					315					320	
cga	aat	gcc	acg	gta	gaa	atg	aca	aga	agg	cat	ata	ı gga	aga	gga	gtg	1008
Arg	Asn	Ala	Thr	Val	Glu	Met	Thr	Arg	Arg	His	Ile	Gly	Arç	Gly	/ Val	
				325					330	•				335	5	
cgc	tta	tac	tac	ata	ggt	ggg	gaa	gtt	. ttt	gct	gaç	g tgc	cta	agt	gat	1056
Arg	Leu	Tyr	Туг	Ile	Gly	Gly	Glu	Val	. Phe	e Ala	Glu	ı Cys	Let	sei	Asp	
			340)				345	;				350)		
agt	gca	atc	ttt	gtg	cag	ago	ccc	aat	tgt:	. aat	caq	g aga	tat	gg	c tgg	1104
Ser	Ala	Ile	Phe	val	Glr	Ser	Pro) Asr	ъ Суз	s Asr	ı Glı	n Arc	ј Ту	r Gl	y Trp	
		355	•				360)				365	5			
cac	cct	gca	aca	a gtg	, tgt	. aaa	att	c cca	a cca	a ggo	c tg	t aat	cto	g aa	g atc	1152
His	Pro	Ala	Thi	val	. Суз	ь Гр	: I1e	e Pro	Pro	o Gly	у Су	s Ası	ı Le	u Ly	s Ile	
	370)				375	5				38	0				
ttc	aac	aac	caq	g gaa	a ttt	c gct	gct	t cti	t ct	g gct	t ca	g tc	t gt	t aa	t cag	1200

Phe 385	Asn	Asn	Gln	Glu	Phe 390	Ala	Ala	Leu	Leu	Ala 395	Gln	Ser	Val	Asn	Gln 400	
ggt	ttt	gaa	gcc	gtc	tat	cag	cta	act	aga	atg	tgc	acc	ata	aga	atg	1248
Gly	Phe	Glu	Ala	Val	Tyr	Gln	Leu	Thr	Arg	Met	Суѕ	Thr	Ile	Arg	Met	
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Ser	Phe	Val	Lys	Gly	Trp	Gly	Ala	Glu	Tyr	Arg	Arg	Gln	Thr	Val	Thr	
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Ser	Thr	Pro	Суѕ	Trp	Ile	Glu	Leu	His	Leu	Asn	Gly	Pro	Leu	Gln	Trp	
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ttg	gac	aaa	gta	tta	act	cag	atg	gga	tcc	cct	tca	gtg	cgt	tgc	tca	1392
Leu	Asp	Lys	Val	Leu	Thr	Gln	Met	Gly	Ser	Pro	Ser	Val	Arg	Cys	Ser	
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agc	atg	tca	tgg	gta	ccg	cgg	gcc	cgg	gat	cca	ccg	gtc	gcc	acc	atg	1440
		Ser	Trp	Val	Pro	Arg	Ala	Arg	Asp			Val	Ala	Thr		
465					470					475					480	
_	=	_				_								_	gtc	1488
val	Ser	Lys	GLy		Glu	Leu	Phe	Thr	-		. Val	Pro	He			
				485					490					495		1536
_	_	-		-	-				_		-				gag	1536
Glu	Leu	Asp	_		val	Asn	GTA			Pne	e Ser	· Vai			Glu	
			500					505				-	510			
												-			: tgc	1584
GLy	Glu	_	_	> Ala	Thr	Tyr		_	Let	Thi	Lev	_		: Ile	e Cys	
		515	i				520)				525	•			
								_							ctg	1632
Thr			Lys	s Lev	ı Pro			Tr	Pro	Thi			Thi	Th:	c Leu	
	530	•				535	5				540)				
acc	: tac	ggc	gto	g cag	g tgc	: ttc	ago	c cg	tac	000	c gad	cac	c ato	g aaq	g cag	1680

Thr 545	Tyr	Gly	Val	Gln	Cys 550	Phe	Ser	Arg	Tyr	Pro 555	Asp	His	Met	Lys	Gln 560	
343					330					333	•				500	
cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	cgc	1728
His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	Arg	
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acc	atc	ttc	ttc	aaq	gac	gac	aac	aac	tac	aaq	acc	cqc	acc	gag	ata	1776
					_	_				_		Arg	-		-	
			580	-	•	-	-	585	_	_		-	590			
aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	atc	1824
Lys	Phe		Gly	Asp	Thr	Leu		Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile	
		595					600					605				
gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	aac	1872
Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn	
	610					615					620					
		-			-			-	-	_	-	_	_		ggc	1920
_		Ser	His	Asn		Tyr	Ile	Met	Ala	_	_	Gln	Lys	Asn	Gly	
625					630					635					640	
atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	: atc	gag	g gac	ggc	ago	gtg	1968
Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	Val	
				645	i				650)				655	5	
_		_	_			_	_						_		ccc	2016
Gin	Leu	ı Ala	_		Tyr	GIn	Glr			: Pro) 116	e GIĀ			y Pro	
			660					665)				670	,		
gtg	; ctg	, cto	g ccc	gac	c aac	cac	: tac	cto	g ago	e acc	cac	g too	gco	ct	g agc	2064
Val	. Leu	Let	ı Pro	Asp	Asr.	His	туз	: Le	ı Sei	Th:	Gli	n Ser	: Ala	a Le	u Ser	
		675	5				680)				685	5			
	-						-					-	-	-	c gtg	2112
гЛs	8 Asp 690) AST	1 611	r rλs	695 695		י או	s Me	ı va.	тье [.] 70		ו הדו	ı Pn	e Val	
	יצט	,				U J	,				, (U				
aco	e geo	c gc	s ggg	g ato	c act	cto	c gg	c at	g ga	c ga	g ct	g ta	c aa	g ta	a	2157

35

Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys * 705 710 715

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<213> Aequorea victoria and human

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Cys Arg Leu Trp Arg Trp Pro Asp Leu His Ser His His Glu Leu Lys
130 135 140

Ala Ile Glu Asn Cys Glu Tyr Ala Phe Asn Leu Lys Lys Asp Glu Val 145 150 155 160

Cys Val Asn Pro Tyr His Tyr Gln Arg Val Glu Thr Pro Val Leu Pro

165 170 175

Pro Val Leu Val Pro Arg His Thr Glu Ile Leu Thr Glu Leu Pro Pro 180 185 190

Leu Asp Asp Tyr Thr His Ser Ile Pro Glu Asn Thr Asn Phe Pro Ala 195 200 205

Gly Ile Glu Pro Gln Ser Asn Tyr Ile Pro Glu Thr Pro Pro Pro Gly
210 215 220

Tyr Ile Ser Glu Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser 225 230 235 240

Met	Asp	Thr	Gly	Ser	Pro	Ala	Glu	Leu	Ser	Pro	Thr	Thr	Leu	Ser	Pro
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Val	Asn	His	Ser	Leu	Asp	Leu	Gln	Pro	Val	Thr	Tyr	Ser	Glu	Pro	Ala
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Phe	Trp	Cys	Ser	Ile	Ala	Tyr	Tyr	Glu	Leu	Asn	Gln	Arg	Val	Gly	Glu
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Thr	Phe	His	Ala	Ser	Gln	Pro	Ser	Leu	Thr	Val	Asp	Gly	Phe	Thr	Asp
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Pro	Ser	Asn	Ser	Glu	Arg	Phe	Суз	Leu	Gly	Leu	Leu	Ser	Asn	Val	Asn
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Arg	Asn	Ala	Thr	Val	Glu	Met	Thr	Arg	Arg	His	Ile	Gly	Arg	Gly	Val
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Arg	Leu	Tyr	Tyr	Ile	Gly	Gly	Glu	Val	Phe	Ala	Glu	Cys	Leu	Ser	Asp
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Ser	Ala	Ile	Phe	Val	Gln	Ser	Pro	Asn	Суѕ	Asn	Gln	Arg	Tyr	Gly	Trp
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His	Pro	Ala	Thr	Val	Cys	Lys	Ile	Pro	Pro	Gly	Cys	Asn	Leu	Lys	Ile
	370					375					380				
Phe	Asn	Asn	Gln	Glu	Phe	Ala	Ala	Leu	Leu	Ala	Gln	Ser	Val	Asn	Gln
385					390					395					400
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Ser	Phe	Val	Lys	Gly	Trp	Gly	Ala	Glu	Tyr	Arg	Arg	Gln	Thr	Val	Thr
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Ser	Thr	Pro	Cys	Trp	Ile	Glu	Leu	His	Leu	Asn	Gly	Pro	Leu	Gln	Trp
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Leu	Asp	Lys	Val	Leu	Thr	Gln	Met	Gly	Ser	Pro	Ser	Val	. Arg	Cys	Ser
	450					455					460				
		Ser	Trp	Val		_	Ala	Arg	Asp			Val	. Ala	Thr	Met
465					470					475					480
Val	Ser	Lys	: Gly			Leu	ı Phe	Thr			. Val	. Pro) Ile		ı Val
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Glu	ı Lev	a Asp			Val	. Asn	Gly			? Phe	e Ser	· Val			7 Glu
			500					505					510		
Gl y	, Glu) Ala	1 Thr	Туг			: Le	ı Thr	Let			e Ile	e Cys
_		515					520					525			_
Thr			y Lys	s Let	ı Pro			Trp	Pro	Thr			l Th:	r Thi	c Leu
	530					535					540				
		Gly	y Val	l Glı			e Sei	r Arg	y Ty:			o His	s Me	t Lys	s Gln
510					550	٦				555	ξ.				560

His	Asp	Phe	Phe		Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln		Arg	
			_,	565	_	_	03	.	570	Ţ.		70.	7.1 .	575	**- 7	
Thr	Ile	Phe	Phe 580	Lys	Asp	Asp	GIÀ	585	Tyr	Lys	Thr	Arg	590	Glu	vai	
Lys	Phe	Glu 595	Gly	Asp	Thr	Leu	Val 600	Asn	Arg	Ile	Glu	Leu 605	Lys	Gly	Ile	
Asp			Glu	Asp	Gly			Leu	Gly	His	-		Glu	Tyr	Asn	
_	610		,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		77. 7	615	T3 -	N - 4	2.1	7	620	<i>α</i> 3.	.	7	C1	
	Asn	Ser	HIS	Asn		Tyr	116	Met	Ala		ьуs	GIN	ьys	Asn		
625	T	**- 3	7	D)	630	T1 -	7)	11.5 -	70	635	G1	2	C1	C	640	
11e	ьуs	vaı	Asn	645	ьуѕ	116	Arg	nis	Asn 650	rre	GIU	Asp	GТÀ	5er 655	Vai	
Gln	Leu	Ala	Asp 660	His	Tyr	Gln	Gln	Asn 665	Thr	Pro	Ile	Gly	Asp 670		Pro	
Val	Leu	Leu 675		Asp	Asn	His	Tyr 680		Ser	Thr	Gln	Ser 685			Ser	
Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val	
	690					695					700					
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Met	Val	. Ser	Lys	: Gl)	g Glu	ı Glı	ı Lev	ı Phe	€ Thi	Gly	y Val	l Val	l Pro	o Ile	e Leu	
1				5					10)				1!	5	
gtc	gaç	, ctç	gac	gg	gad	gta	a aac	c ggo	c cad	c aag	g tt	c ago	c gt	g tc	c ggc	96
Val	Glu	ı Lev	ı Asp	Gly	/ Asp	va:	l Ası	n Gly	y His	s Ly:	s Ph	e Sei	r Va	l Se	r Gly	
			20)				25	5				3	0		
gag	i aac	: gad	ı aac	r dai	r aca	c acc	c tad	ב ממנ	саас	a cti	ന കര	o ata	or aa	a tt	c atc	144

Glu	Gly	G1u 35	GIA	Asp	Ala	Thr	Tyr 40	GIÀ	гàг	Leu	Thr	ьеи 45	ьуs	Phe	IIe	
_				-	-			ccc Pro								192
.,	50		4			55			•		60					
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Tyr	Gly	Val	Gln	Суѕ	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
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cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val		Glu	
				85					90					95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys			Gly	Asp	Thr		Val	Asn	Arg	Ile		Leu	Lys	Gly	
		115					120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
	130	ŧ				135					140					
aac	tac	aac	ago	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480
Asn	Туг	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155					160	
ggc	ato	aag	gtg	aac	ttc	aag	ato	cgc	cac	aac	ato	gag	gac	ggc	agc	528
Gly	Ile	Lys	Val	. Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	e Glu	. Asp	Gly	Ser	
				165	•				170	1				175		
gtg	cag	g etc	gcc	gac	: cac	tac	: cag	r cag	aac	acc		ato	ggo	gac	ggc:	57€
Val	Glr	ı Lev	a Ala	a Asp	His	Tyr	Glr	Gln	Asr	Thi	Pro	o Ile	e Gly	y Asp	Gly	
			180)				185	i				190)		
ccc	gt q	g cto	g cto	g ccc	gac	aac	cac	: tac	cto	, ago	cac	caq	g tco	e ged	ctg	624

Pro	Val	Leu 195	Leu	Pro	Asp	Asn	His 200	Tyr	Leu	Ser	Thr	Gln 205	Ser	Ala	Leu	
agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	
	210					215					220					
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag	tcc	720
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Ser	
225					230					235					240	
		-		-	-		_		_	-		acg	-			768
Gly	Leu	Arg	Ser		Ala	Gln	Ala	Ser		Ser	Glu	Thr	Val		Met	
				245					250					255		
agc	gag	acg	gtc	atc	tgt	tcc	agc	cgg	gcc	act	gtg	atg	ctt	tat	gat	816
Ser	Glu	Thr		Ile	Суѕ	Ser	Ser	_	Ala	Thr	Val	Met		Tyr	Asp	
			260					265					270			
-				-				_				ccc	_	-		864
Asp	Gly		-	Arg	Trp	Leu			Gly	Thr	Gly	Pro		Ala	Phe	
		275					280					285				010
		-	_												gtc	912
Ser	Arg 290		GIN	ire	Tyr	295		Pro	inr	Ala	300	Ser	Pne	Arg	vai	
~+ ~														. +~+		960
			_	_	_		•	_	_		-			_	gcc	900
305	-	Arg	гуз	met	310		ASP	011.	611	315		. 116	: ASI	г суз	320	
																1000
	_			_	_			_	_						cag	1008
lle	Val	Arg	l GTA		-	Тут	Asn	ı Gir			Pro	o Asr	Phe		Gln	
				325)				330	J				335)	
	_	_	_	_	_	-							_		g gag	1056
Trp	Arg) Asp			g Glr	val	Trp			ı Asr) Ph	e Gly			s Glu	
			340)				345	Ď				356	IJ		
gat	ggg	g gcc	cag	j tti	ged	geo	gg(c ato	g gc	c agt	gc	c cta	a ga	g gc	g ttg	1104

Asp	Ala	Ala 355	Gln	Phe	Ala	Ala	Gly 360	Met	Ala	Ser	Ala	Leu 365	Glu	Ala	Leu	
_					cct Pro									_		1152
					ccg Pro 390											1200
		_	-	-	cac His		-	-		-			-	• -		1248
					gct Ala									Pro		1296
			Gly		ccc			Pro					Ser			1344
	-	Ala			gga Gly	_	Gly					Pro	_		cct	1392
	Pro					Pro					/ Ala				ggc Gly 480	1440
					Ala					a Arg					g cag s Gln	1488
				Gly					a Pro					r Gl	cga Y Arg	1536
ago	gga	a ggt	c ggg	g gga	a cto	ato	g gaa	a ga	g ato	g aa	c gc	c ato	g ct	g gc	c cgg	1584

41

Ser Gly Gly Gly Leu Met Glu Glu Met Asn Ala Met Leu Ala Arg 515 520 525

aga agg aaa gcc acg caa gtt ggg gag aaa acc ccc aag gat gaa tct 1632
Arg Arg Lys Ala Thr Gln Val Gly Glu Lys Thr Pro Lys Asp Glu Ser
530 535 540

gcc aat cag gag gag cca gag gcc aga gtc ccg gcc cag agt gaa tct 1680

Ala Asn Gln Glu Glu Pro Glu Ala Arg Val Pro Ala Gln Ser Glu Ser

550 550 550

gtg cgg aga ccc tgg gag aag aac agc aca acc ttg cca agg atg aag 1728

Val Arg Arg Pro Trp Glu Lys Asn Ser Thr Thr Leu Pro Arg Met Lys

565 570 575

teg tet tet teg gtg ace act tee gag ace caa eee tge acg eee age 1776

Ser Ser Ser Val Thr Thr Ser Glu Thr Gln Pro Cys Thr Pro Ser
580 585 590

tcc agt gat tac tcg gac cta cag agg gtg aaa cag gag ctt ctg gaa 1824 Ser Ser Asp Tyr Ser Asp Leu Gln Arg Val Lys Gln Glu Leu Leu Glu 595 600 605

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Glu	Gly	Glu 35	Gly	Asp	Ala	Thr	Tyr 40	Gly	Lys	Leu	Thr	Leu 45	Lys	Phe	Ile
Cys	Thr 50	Thr	Gly	Lys	Leu	Pro 55	Val	Pro	Trp	Pro	Thr 60	Leu	Val	Thr	Thr
Leu 65	Thr	Tyr	Gly	Val	Gln 70	Cys	Phe	Ser	Arg	Tyr 75	Pro	Asp	His	Met	Lys 80
Gln	His	Asp	Phe	Phe 85	Lys	Ser	Ala	Met	Pro 90	Glu	Gly	Tyr	Val	Gln 95	Glu
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly 105	Asn	Tyr	Lys	Thr	Arg	Ala	Glu
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu 120	Val	Asn	Arg	Ile	Glu 125	Leu	Lys	Gly
Ile	Asp 130	Phe	Lys	Glu	Asp	Gly 135	Asn	Ile	Leu	Gly	His 140	Lys	Leu	Glu	Tyr
Asn 145	Tyr	Asn	Ser	His	Asn 150	Val	Tyr	Ile	Met	Ala 155	Asp	Lys	Gln	Lys	Asn 160
Gly	Ile	Lys	Val	Asn 165	Phe	Lys	Ile	Arg	His 170	Asn	Ile	Glu	Asp	Gly 175	Ser
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Pro	Val	Leu 195	Leu	Pro	Asp	Asn	His 200	Tyr	Leu	Ser	Thr	Gln 205	Ser	Ala	Leu
Ser	Lys 210	Asp	Pro	Asn	Glu	Lys 215	_	Asp	His	Met	Val 220		Leu	Glu	Phe
Val 225		Ala	Ala	Gly	11e 230	Thr	Leu	Gly	Met	Asp 235		Leu	Tyr	Lys	Ser 240
Gly	Leu	Arg	Ser	Arg 245	Ala	Gln	Ala	Ser	Met 250		Glu	Thr	· Val	Ile 255	
Ser	Glu	Thr	Val 260		Cys	Ser	Ser	Arg 265		Thr	Val	Met	Leu 270		Asp
Asp	Gly	Asn 275	-	Arg	Trp	Leu	Pro 280		Gly	Thr	Gly	Pro 285	Gln	Ala	Phe
Ser	Arg 290		Gln	Ile	Tyr	His 295		Pro	Thr	Ala	Asr 300		Phe	e Arg	Val
Val		Arg	Lys	: Met	Gln 310		Asp	Gln	Gln	Val		. Ile	e Asn	Cys	Ala 320
Ile	val	. Arg	Gly	7 Val		Tyr	Asn	Gln	Ala 330		Pro	Ası	n Phe	His 335	

Trp	Arg	Asp	Ala	Arg	Gln	Val	Trp	Gly	Leu	Asn	Phe	Gly	Ser	Lys	Glu
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Asp	Ala	Ala	Gln	Phe	Ala	Ala	Gly	Met	Ala	Ser	Ala	Leu	Glu	Ala	Leu
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Glu	Gly	Gly	Gly	Pro	Pro	Pro	Pro	Pro	Ala	Leu	Pro	Thr	Trp	Ser	Val
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Pro	Asn	Gly	Pro	Ser	Pro	Glu	Glu	Val	Glu	Gln	Gln	Lys	Arg	Gln	Gln
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Pro	Gly	Pro	Ser	Glu	His	Ile	Glu	Arg	Arg	Val	Ser	Asn	Ala	Gly	Gly
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Pro	Pro	Ala	Pro	Pro	Ala	Gly	Gly	Pro	Pro	Pro	Pro	Pro	Gly	Pro	Pro
			420					425					430		
Pro	Pro	Pro	Gly	Pro	Pro	Pro	Pro	Pro	Gly	Leu	Pro	Pro	Ser	Gly	Val
		435					440					445			
Pro	Ala	Ala	Ala	His	Gly	Ala	Gly	Gly	Gly	Pro	Pro	Pro	Ala	Pro	Pro
	450					455					460				
Leu	Pro	Ala	Ala	Gln	Gly	Pro	Gly	Gly	Gly	Gly	Ala	Gly	Ala	Pro	Gly
465					470					475					480
Leu	Ala	Ala	Ala	Ile	Ala	Gly	Ala	Lys	Leu	Arg	Lys	Val	Ser	Lys	Gln
				485					490					495	
Glu	Glu	Ala	Ser	Gly	Gly	Pro	Thr	Ala	Pro	Lys	Ala	Glu	Ser	Gly	Arg
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Ser	Gly	Gly	Gly	Gly	Leu	Met	Glu	Glu	Met	Asn	Ala	Met	Leu	Ala	Arg
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Arg	Arg	Lys	Ala	Thr	Gln	Val	Gly	Glu	Lys	Thr	Pro	Lys	Asp	Glu	Ser
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Ala	Asn	Gln	Glu	Glu	Pro	Glu	Ala	Arg	Val	Pro	Ala	Gln	Ser	Glu	Ser
545					550					555					560
Val	Arg	Arg	Pro	Trp	Glu	Lys	Asn	Ser	Thr	Thr	Leu	Pro	Arg	Met	Lys
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Ser	Ser	Ser	Ser	Val	Thr	Thr	Ser	Glu	Thr	Gln	Pro	Cys	Thr	Pro	Ser
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Ser	Ser	Asp	Tyr	Ser	Asp	Leu	Gln	Arg	Val	Lys	Gln	Glu	Leu	Leu	Glu
		595	,				600					605	•		
Glu	Val	Lys	. Lys	Glu	Leu	Gln	Lys	Val	. Lys	: Glu	Glu	Ile	: Ile	Glu	Ala
	610)				615					620	i			
Phe	· Val	. Glr	ı Glu	Let	Arg	Lys	Arg	Gly	7 Ser	Pro	1				
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44

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1 5 10 15

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Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly

20 25 30

gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile

35

40

45

tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr

50 55 60

ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys

65 70 75 80

cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu

85

90

95

cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag 336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100 105 110

gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly

115

120

125

atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	Ĥis	Lys	Leu	Glu	Tyr	
	130					135					140					
								atc								480
Asn	Tyr	Asn	Ser	His		Val	Tyr	Ile	Met		Asp	Lys	Gln	Lys		
145					150					155					160	
aac	atc	220	ata	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
								Arg								
GIY	110	טעט	V G L	165		-1-			170				_	175		
gtg	cag	cto	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	lle	Gly	Asp	Gly	
			180					185					190	1		
																50.1
															c ctg	624
Pro	Val			Pro	Asp	Asn			Leu	Ser	Thi			: Ala	a Leu	
		195	5				200)				205)			
- ~			2 000		- 426	T 220	cac	r dat	cac	: ato	ı atı	e ete	r cto	a da	g ttc	672
															u Phe	
001	210					215					22					
gt	g ac	c gc	c gc	c gg	g at	c act	ct	c ggc	ato	g ga	c ga	g ct	g ta	c aa	g tcc	720
Va.	1 Th	r Al	a Ala	a Gl	y Il	e Thi	. Le	u Gly	/ Met	. As	o Gl	u Le	u Ty	r Ly	s Ser	•
22	5				23	0				23	5				240)
																7.00
															c ccc	
G1	y Le	u Ar	g Se			a Me	t As	p Glı			e Pr	o Le	u II		ne Pro	>
				24	5				25	U				25))	
~~	.a. «a	a cc			ים מכ	r to	t aa	ות ממו	c ta	t at	a aa	ag at	c at	it qa	ag caq	g 816
															lu Gli	
***	. u 01		26					26					27			
				-												
cc	c aa	ıg ca	ag co	gg gg	gc at	g cg	c tt	c cg	c ta	c aa	g to	gc ga	ag g	gg c	gc tc	c 864
															rg Se	
		2	75				28	30				28	35			

			atc														912
Ala	Gly	Ser	Ile	Pro	Gly	Glu	Arg	Ser	Thr	Asp	Thr	Thr	Lys	Thr	H	is	
	290					295					300						
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			aag														960
Pro	Thr	Ile	Lys	Ile		Gly	Tyr	Thr	Gly		Gly	Thr	Vai	Arg			
305					310					315					-	320	
														~~.	~ ~	-++	1008
			acc														1000
Ser	Leu	Val	Thr		Asp	Pro	Pro	HIS		Pro	HIS	Pro	nıs	33		Leu	
				325					330					٠,	J		
			gac	+ ~ ~	222	ast	aac	ttc	tat	gag	act	nan	cto	: t.a	c (cca	1056
_			gac														
vaı	СТА	ьуѕ	340	Суѕ	Arg	ASP	Gry	345		014	****	- 020	350				
			340					313									
asc	cac	· tac	atc	cac	aat	ttc	cag	aac	cta	gga	ato	cac	ı tg	gt.	g	aag	1104
-			. Ile														
1101	, 111	355					360			_		365					
aac	g ege	g gad	c ctg	gag	g cag	gct	ato	: agt	cag	, cgc	ato	c ca	g ac	c aa	ac	aac	1152
			o Lev														
	370)				375	ı				380	0					
aad	2 00	c tt	c caa	a gtt	t cct	ata	gaa	a gaç	g caq	g cg	t gg	g ga	c ta	c ga	ac	ctg	1200
Ası	n Pr	o Ph	e Glr	ı Va	l Pro	o Ile	e Glu	ı Glu	ı Glı	n Ar	g Gl	y As	р Ту	r A	sp	Leu	
38	5				390)				39	5					400	
			g cg														1248
As	n Al	a Va	1 Ar	g Le	u Cy	s Phe	e Gl	n Va	1 Th	r Va	l Ar	g As	p Pi	o S	er	Gly	
				40	5				41	0				4	15		
			c cg														1296
Ar	g Pr	o Le	u Ar	g Le	u Pr	o Pr	o Va	1 Le	u Pr	o Hi	s Pr	o II			sp	Asn	
			42	0				42	:5				4	30			
																	1744
																aac	1344
Ar	g Al			n Th	nr Al	a Gl			s Il	.e Cy	ys Ai			sn A	4rç	g Asn	
		4.	35				44	0				4	45				

tct	ggc	agc	tgc	ctc	ggt	ggg	gat	gag	atc	ttc	cta	ctg	tgt	gac	aag	1392
Ser	Gly	Ser	Cys	Leu	Gly	Gly	Asp	Glu	Ile	Phe	Leu	Leu	Cys	Asp	Lys	
	450					455					460					
	cag		5 5	-						_						1440
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465					470					475					480	
acc	cga	aac	tcc	+++	tca	caa	act	ast	ata	Cac	cas	caa	ata	acc	2tt	1488
_	Arg				_		_	_			_			_		1400
712.0	mg	CLY	DCL	485	DCI	OIII	mu	пор	490	111.5	nig	GIN	Val	495	110	
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gtg	ttc	cgg	acc	cct	ccc	tac	gca	gac	ccc	agc	ctg	cag	gct	cct	gtg	1536
Val	Phe	Arg	Thr	Pro	Pro	Tyr	Ala	Asp	Pro	Ser	Leu	Gln	Ala	Pro	Val	
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Arg	Val	Ser	Met	Gln	Leu	Arg	Arg	Pro	Ser	Asp	Arg	Glu	Leu	Ser	Glu	
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	_	_		_		_		-		-	-	_			att 	1632
Pro		Glu	Phe	GIn	Tyr			Asp	Thr	Asp	-	_	His	Arg	Ile	
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gag	gag	aaa	cat	aaa	agg	aca	tat	gag	acc	ttc	aaq	ago	ato	ato	aaq	1680
			_								_				Lys	
545		-2-	9	-1-	550		-1-			555	-				560	
aag	agt	cct	ttc	ago	gga	ccc	acc	gac	ccc	cgg	cct	cca	cct	. cga	cgc	1728
Lys	Ser	Pro	Phe	Ser	Gly	Pro	Thr	Asp	Pro	Arg	Pro	Pro	Pro	Arg	Arg	
				565	ı				570	ı			-	575	5	
att	gct	gtg	cct	tcc	: cgc	ago	tca:	gct	tct	gto	ccc	aaç	cca	a gca	ccc	1776
Ile	Ala	Val	. Pro	Ser	Arg	Ser	Ser	Ala	Ser	Val	. Pro	Lys	Pro	Ala	a Pro	
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															gag	1824
Gln	Pro			Ph∈	e Thr	Ser			ı Ser	Thi	: Ile		_	c Asp	o Glu	
		595)				600)				605	ō			

ttt	ccc	acc	atg	gtg	ttt	cct	tct	ggg	cag	atc	agc	cag	gcc	tcg	gcc	1872
Phe	Pro	Thr	Met	Val	Phe	Pro	Ser	Gly	Gln	Ile	Ser	Gln	Ala	Ser	Ala	
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Leu	Ala	Pro	Ala	Pro	Pro	Gln	Val	Leu	Pro	Gln	Ala	Pro	Ala	Pro	Ala	
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cct	gct	cca	gcc	atg	gta	tca	gct	ctg	gcc	cag	gcc	cca	gcc	cct	gtc	1968
Pro	Ala	Pro	Ala	Met	Val	Ser	Ala	Leu	Ala	Gln	Ala	Pro	Ala	Pro	Val	
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cca	gtc	cta	gcc	cca	ggc	cct	cct	cag	gct	gtg	gcc	cca	cct	gcc	ccc	2016
Pro	Val	Leu	Ala	Pro	Gly	Pro	Pro	Gln	Ala	Val	Ala	Pro	Pro	Ala	Pro	
			660					665					670			
٠																
aag	ccc	acc	cag	gct	ggg	gaa	gga	acg	ctg	tca	gag	gcc	ctg	ctg	cag	2064
Lys	Pro	Thr	Gln	Ala	Gly	Glu	Gly	Thr	Leu	Ser	Glu	Ala	Leu	Leu	Gln	
		675					680					685				
ctg	cag	ttt	gat	gat	gaa	gac	ctg	ggg	gcc	ttg	ctt	ggc	aac	ago	aca	2112
Leu	Gln	Phe	Asp	Asp	Glu	Asp	Leu	Gly	Ala	Leu	Leu	Gly	Asn	Ser	Thr	
	690					695					700)				
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Asp	Pro	Ala	Val	Phe	Thr	Asp	Leu	Ala	Ser	Val	Asp	Asn	Ser	Glu	ı Phe	
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cag	cag	ctg	, ctg	aac	cag	ggc	ata	cct	gtg	geo	: ccc	cac	aca	act	gag	2208
															Glu	
				725		_			730				-	735		
ccc	atg	cto	, atq	gaq	, tac	cct	. gag	, gct	ata	act	cgo	c cta	gto	g aca	a ggg	2256
	_	_						_			_			-	r Gly	
			740		- 4			745					750		- 4	
qcc	: cac	aaa	7 CCC	ccc	c gad	: cca	ı act	. cct	act	. cca	a cto	a aa	a acc	c cc	g ggg	2304
					-								_		o Gly	
		75	-				760					76	-		1	
		, , ,	-				, 50	-				, 0.	-			

ctc ccc aat ggc ctc ctt tca gga gat gaa gac ttc tcc tcc att gcg 2352
Leu Pro Asn Gly Leu Leu Ser Gly Asp Glu Asp Phe Ser Ser Ile Ala
770 775 775 780

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Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu
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Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	∀al	Leu	Leu	Glu	Phe
210					215					220				
Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Ser
				230					235					240
Leu	Arg	Ser	Arg	Ala	Met	Asp	Glu	Leu	Phe	Pro	Leu	Ile	Phe	Pro
			245					250					255	
Glu	Pro	Ala	Gln	Ala	Ser	Gly	Pro	Tyr	Val	Glu	Ile	Ile	Glu	Gln
		260					265					270		
Lys	Gln	Arg	Gly	Met	Arg	Phe	Arg	Tyr	Lys	Cys	Glu	Gly	Arg	Ser
	275					280					285			
Gly	Ser	Ile	Pro	Gly	Glu	Arg	Ser	Thr	Asp	Thr	Thr	Lys	Thr	His
					295					300				
Thr	Ile	Lys	Ile		Gly	Tyr	Thr	Gly	Pro	Gly	Thr	Val	Arg	Ile
_									315					320
Leu	Val	Thr		Asp	Pro	Pro	His		Pro	His	Pro	His		Leu
	_	_												
GIY	Lys		Cys	Arg	Asp	Gly		Tyr	Glu	Ala	Glu		Суѕ	Pro
7				_				_						_
Arg		11e	His	Ser	Phe		Asn	Leu	GIY	He		Cys	Val	Lys
7) *~~		Ton	C1	C1 n	71.		Co.~	C1-	71	т1.		m)	70	7
	Asp	ьеи	GIU	GIII		Tre	ser	GIII	Arg		GIU	Thr	Asn	ASI
	Phe	Gln	f e W	Pro		Clu	Clu	Cln	71 20 00		7.00	Ф	7.00	T 0.11
110	1110	0111	Val		116	Giu	Giu	GIII		СТУ	АЗР	TAT	Asp	400
Ala	Val	Ara	Leu		Phe	Gln	Val	Thr		Ara	Asn	Pro	Ser	
		9				01				9	пор	110		
Pro	Leu	Ara			Pro	Val	Len			Pro	Tle	Phe		
		420												11011
Ala	Pro	Asn	Thr	Ala	Glu	Leu	Lys	Ile	Cvs	Ara	Val		Ara	Asn
	435					440	•		_	,				
Gly	Ser	Cys	Leu	Gly	Gly	Asp	Glu	Ile	Phe	Leu			Asp	Lvs
		_		_								_	-	_
Gln	Lys	Glu	Asp	Ile	Glu	Val	Tyr	Phe	Thr	Gly	Pro	Gly	Trp	Glu
							_					-	-	480
Arg	Gly	Ser	Phe	Ser	Gln	Ala	Asp	Val	His	Arg	Gln	Val	Ala	Ile
							-			-			495	
Phe	Arg	Thr	Pro	Pro	Tyr	Ala	Asp	Pro	Ser	Leu	Gln	Ala	Pro	Val
	Lys 210 Thr Leu Glu Lys Gly 290 Thr Leu Gly Arg Arg 370 Pro Ala Pro Ala Gly 450 Gln Arg	Lys Asp 210 Thr Ala Leu Arg Glu Pro Lys Gln 275 Gly Ser 290 Thr Ile Leu Val Gly Lys Arg Cys 355 Arg Asp 370 Pro Phe Ala Val Pro Leu Ala Pro Ala Pro 435 Gly Ser 450 Gln Lys	Lys Asp Pro 210 Pro Thr Ala Ala Leu Arg Ser Glu Pro Ala 260 Arg Arg Lys Gln Arg 275 Ile Lys Gly Ser Ile 290 Asp Asp Leu Val Thr Gly Asp Leu 370 Pro Arg Pro Phe Gln Ala Val Arg 420 Ala Pro Ala Pro Asn 435 Cys 450 Arg Cys 4	Lys Asp Pro Asn 210 Thr Ala Gly Thr Ala Ala Gly Leu Arg Ala Gln 260 245 Glu Pro Ala Gln 260 275 Pro Gly Ser Ile Pro 290 Thr Lys Ile Leu Val Thr Lys Gly Lys Asp Cys 340 Arg Cys Arg Asp Cys 340 His Arg Asp Cys 340 His Arg Asp Cys Arg Asp Cys Arg Asp Leu 405 Arg Leu 4	Lys Asp Pro Asn Glu 210 Thr Ala Ala Gly Ile Thr Ala Ala Gly Ile 230 Leu Arg Ser Arg Ala 245 Glu Pro Ala Gly Ala 245 Glu Pro Ala Gly Ala A	Lys Asp Pro Asn Glu Lys 210	195	195	195	195	195	Lys Asp Pro Asn Glu Lys Asp Asp Het Vex Lyu Lys Asp Asp Het Vex Lyu Lyu Asp Asp Het Vex Lyu Leu Pro Lyu Leu Lyu Lyu <td> 195</td> <td> The transfer of the transfer of transfer</td>	195	The transfer of the transfer of transfer

51

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Pro	Met	Glu	Phe	Gln	Tyr	Leu	Pro	Asp	Thr	Asp	Asp	Arg	His	Arg	Ile
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545					550					555					560
Lys	Ser	Pro	Phe	Ser	Gly	Pro	Thr	Asp	Pro	Arg	Pro	Pro	Pro	Arg	Arg
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Ile	Ala	Val	Pro	Ser	Arg	Ser	Ser	Ala	Ser	Val	Pro	Lys	Pro	Ala	Pro
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	Ala	Pro	Ala	Pro		Gln	Val	Leu	Pro		Ala	Pro	Ala	Pro	Ala
625		_			630	_		_		635					640
Pro	Ala	Pro	Ala		Val	Ser	Ala	Leu		Gln	Ala	Pro	Ala		Val
Dwa	777	T	7. 7	645	G1	D	D	03	650		** 7	_	_	655	_
PLO	val	ьeu	660	Pro	ату	Pro	Pro	Gln 665	АТа	vaı	Ата	Pro		Ата	Pro
T.ve	Pro	Thr		Δla	Glu	Glu	Glu	Thr	Tou	Sor	Clu	70.70	670	T 011	C1~
11,75	110	675	GIII	Ald	GLY	GIU	680	1111	Deu	Ser	Giu	685	Leu	rea	GIII
Leu	Gln		Asp	Asp	Glu	Asp		Gly	Ala	Len	T.en		Asn	Ser	Thr
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Asp	Pro	Ala	Val	Phe	Thr		Leu	Ala	Ser	Val		Asn	Ser	Glu	Phe
705					710	_				715	-				720
Gln	Gln	Leu	Leu	Asn	Gln	Gly	Ile	Pro	Val	Ala	Pro	His	Thr	Thr	Glu
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Pro	Met	Leu	Met	Glu	Tyr	Pro	Glu	Ala	Ile	Thr	Arg	Leu	Val	Thr	Gly
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Ala	Gln	Arg	Pro	Pro	Asp	Pro	Ala	Pro	Ala	Pro	Leu	Gly	Ala	Pro	Gly
		755					760					765	-		
Leu	Pro	Asn	Gly	Leu	Leu	Ser	Gly	Asp	Glu	Asp	Phe	Ser	Ser	Ile	Ala
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<213>	Aequorea	victoria	and	human
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<220> <221> CDS <222> (1)...(2394) <400> 15 atg gac gaa ctg ttc ccc ctc atc ttc ccg gca gag cca gcc cag gcc Met Asp Glu Leu Phe Pro Leu Ile Phe Pro Ala Glu Pro Ala Gln Ala tct ggc ccc tat gtg gag atc att gag cag ccc aag cag cgg ggc atg Ser Gly Pro Tyr Val Glu Ile Ile Glu Gln Pro Lys Gln Arg Gly Met ege tte ege tae aag tge gag ggg ege tee geg gge age ate eea gge Arg Phe Arg Tyr Lys Cys Glu Gly Arg Ser Ala Gly Ser Ile Pro Gly gag agg agc aca gat acc acc aag acc cac ccc acc atc aag atc aat Glu Arg Ser Thr Asp Thr Thr Lys Thr His Pro Thr Ile Lys Ile Asn ggc tac aca gga cca ggg aca gtg cgc atc tcc ctg gtc acc aag gac Gly Tyr Thr Gly Pro Gly Thr Val Arg Ile Ser Leu Val Thr Lys Asp cet cet cac egg cet cac ece cac gag ett gta gga aag gae tge egg Pro Pro His Arg Pro His Pro His Glu Leu Val Gly Lys Asp Cys Arg gat ggc ttc tat gag gct gag ctc tgc ccg gac cgc tgc atc cac agt Asp Gly Phe Tyr Glu Ala Glu Leu Cys Pro Asp Arg Cys Ile His Ser ttc cag aac ctg gga atc cag tgt gtg aag aag cgg gac ctg gag cag Phe Gln Asn Leu Gly Ile Gln Cys Val Lys Lys Arg Asp Leu Glu Gln get ate agt cag ege ate cag ace aac aac eec tte caa gtt eet

Ala	Ile 130	Ser	Gln	Arg	Ile	Gln 135	Thr	Asn	Asn	Asn	Pro 140	Phe	Gln	Val	Pro		
ata	gaa	gag	cag	cgt	ggg	gac	tac	gac	ctg	aat	gct	gtg	cgg	ctc	tgc	4	80
Ile	Glu	Glu	Gln	Arg	Gly	Asp	Tyr	Asp	Leu	Asn	Ala	Val	Arg	Leu	Cys		
145					150					155					160		
ttc	cag	gtg	aca	gtg	cgg	gac	cca	tca	ggc	agg	ccc	ctc	cgc	ctg	ccg	5	528
Phe	Gln	Val	Thr	Val	Arg	Asp	Pro	Ser	Gly	Arg	Pro	Leu	Arg	Leu	Pro		
				165					170					175			
				cat											_	Ę	576
Pro	Val	Leu		His	Pro	Ile	Phe		Asn	Arg	Ala	Pro	Asn	Thr	Ala		
			180					185					190				
				tgc									_			(624
Glu	Leu	_	Ile	Суѕ	Arg	Val		Arg	Asn	Ser	Gly		Cys	Leu	Gly		
		195					200					205					
				ttc										_		•	672
GIÀ	210	Glu	Ile	Phe	Leu	Leu 215	Cys	Asp	Lys	Val	Gln 220	Lys	Glu	Asp	Ile		
gag	gtg	tat	ttc	acg	gga	сса	ggc	tgg	gag	gcc	cga	ggc	tcc	ttt	tcg		720
Glu	Val	Tyr	Phe	Thr	Gly	Pro	Gly	Trp	Glu	Ala	Arg	Gly	Ser	Phe	Ser		
225					230					235					240		
caa	gct	gat	gtg	cac	cga	caa	gtg	gcc	att	gtg	ttc	cgg	acc	cct	ccc		768
Gln	Ala	Asp	Val	His	Arg	Gln	Val	Ala			Phe	Arg	Thr	Pro	Pro		
				245					250					255			
				agc						_	-		_	_	-		816
Tyr	Ala	Asp		Ser	Leu	Gln	Ala	Pro	Val	Arg	Val	Ser	Met	Gln	Leu		
			260					265					270				
cgg	cgg	cct	tcc	gac	cgg	gag	ctc	agt	gag	ccc	atg	gaa	ttc	cag	tac		864
Arg	Arg			Asp	Arg	Glu	Leu	Ser	Glu	Pro	Met	Glu	Phe	Gln	Tyr		
		275					280	1				285					
ctg	cca	gat	aca	gac	gat	cgt	cac	: cgg	att	gag	gag	aaa	cgt	aaa	agg		912

Leu	Pro 290	Asp	Thr	Asp	Asp	Arg 295	His	Arg	Ile	Glu	Glu 300	Lys	Arg	Lys	Arg	
aca	tat	gag	acc	ttc	aag	agc	atc	atg	aag	aag	agt	cct	ttc	agc	gga	960
Thr	Tyr	Glu	Thr	Phe	Lys	Ser	Ile	Met	Lys	Lys	Ser	Pro	Phe	Ser	Gly	
305					310					315					320	
ccc	acc	gac	ccc	cgg	cct	cca	cct	cga	cgc	att	gct	gtg	cct	tcc	cgc	1008
Pro	Thr	Asp	Pro	Arg	Pro	Pro	Pro	Arg	Arg	Ile	Ala	Val	Pro	Ser	Arg	
				325					330					335		
agc	tca	gct	tct	gtc	ccc	aag	cca	gca	ccc	cag	ccc	tat	ccc	ttt	acg	1056
Ser	Ser	Ala	Ser	Val	Pro	Lys	Pro	Ala	Pro	Gln	Pro	Tyr	Pro	Phe	Thr	
			340					345					350			
tca	tcc	ctg	agc	acc	atc	aac	tat	gat	gag	ttt	ccc	acc	atg	gtg	ttt	1104
Ser	Ser	Leu	Ser	Thr	Ile	Asn	Tyr	Asp	Glu	Phe	Pro	Thr	Met	Val	Phe	
		355					360					365				
cct	tct	ggg	cag	atc	agc	cag	gcc	tcg	gcc	ttg	gcc	ccg	gcc	cct	ccc	1152
Pro	Ser	Gly	Gln	Ile	Ser	Gln	Ala	Ser	Ala	Leu	Ala	Pro	Ala	Pro	Pro	
	370					375					380					
								cct					_	_	_	1200
	Val	Leu	Pro	Gln			Ala	Pro	Ala		Ala	Pro	Ala	Met	Val	
385					390					395					400	
								cct			-		-			1248
Ser	Ala	Leu	Ala		Ala	Pro	Ala	Pro		Pro	Val	Leu	Ala		Gly	
				405					410					415		
													_	_	ggg	1296
Pro	Pro	Gln	Ala	Val	Ala	Pro	Pro	Ala	Pro	Lys	Pro	Thr	Gln	Ala	Gly	
			420					425					430			
gaa	gga	acg	ctg	tca	gag	gcc	ctg	ctg	cag	ctg	cag	ttt	gat	gat	gaa	1344
Glu	Gly	Thr	Leu	Ser	Glu	Ala	Leu	Leu	Gln	Leu	Gln	Phe	Asp	Asp	Glu	
		435					440					445				
gac	ctg	ggg	gcc	ttg	ctt	ggc	aac	agc	aca	gac	cca	gct	gtg	ttc	aca	1392

Asp	Leu 450	Gly	Ala	Leu	Leu	Gly 455	Asn	Ser	Thr	Asp	Pro 460	Ala	Val	Phe	Thr	
gac	ctg	gca	tcc	gtc	gac	aac	tcc	gag	ttt	cag	cag	ctg	ctg	aac	cag	1440
Asp	Leu	Ala	Ser	Val	Asp	Asn	Ser	Glu	Phe	Gln	Gln	Leu	Leu	Asn	Gln	
465					470					475					480	
ggc	ata	cct	gtg	gcc	ccc	cac	aca	act	gag	ccc	atg	ctg	atg	gag	tac	1488
Gly	Ile	Pro	Val	Ala	Pro	His	Thr	Thr	Glu	Pro	Met	Leu	Met	Glu	Tyr	
				485					490					495		
cct	gag	gct	ata	act	cgc	cta	gtg	aca	ggg	gcc	cag	agg	ccc	ccc	gac	1536
Pro	Glu	Ala	Ile	Thr	Arg	Leu	Val	Thr	Gly	Ala	Gln	Arg	Pro	Pro	Asp	
			500					505					510			
cca	gct	cct	gct	cca	ctg	ggg	gcc	ccg	ggg	ctc	ccc	aat	ggc	ctc	ctt	1584
Pro	Ala	Pro	Ala	Pro	Leu	Gly	Ala	Pro	Gly	Leu	Pro	Asn	Gly	Leu	Leu	
		515					520					525				
								att								1632
Ser		Asp	Glu	Asp	Phe		Ser	Ile	Ala	Asp	Met	Asp	Phe	Ser	Ala	
	530					535					540					
								gat								1680
	Leu	Ser	Gln	Ile		Ser	Leu	Asp	Pro		Val	Ala	Thr	Met	Val	
545					550					555					560	
								ggg								1728
Ser	Lys	GTĀ	Glu		Leu	Phe	Thr	Gly		Val	Pro	Ile	Leu		Glu	
				565					570					575		
								aag							-	1776
Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	Glu	Gly	
			580					585					590			
								ctg								1824
Glu	Gly			Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	Cys	Thr	
		595					600					605				
acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	ctg	acc	1872

Thr	Gly 610	Lys	Leu	Pro	Val	Pro 615	Trp	Pro	Thr	Leu	Val 620	Thr	Thr	Leu	Thr	
tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	cag	cac	1920
Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	Gln	His	
625					630					635					640	
					gcc									-		1968
Asp	Phe	Phe	Lys		Ala	Met	Pro	Glu		Tyr	Val	Gln	Glu	_	Thr	
				645					650					655		
atc	ttc	ttc	aad	nac	gac	aac	aac	tac	aad	200	cac	acc	~~~	at a	224	2016
					Asp									-	_	2010
			660		- T	1		665	2,70		9	1114	670	vai	БуЗ	
													• • •			
ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	atc	gac	2064
Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile	Asp	
		675					680					685				
					aac						_					2112
Phe		Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn	Tyr	
	690					695					700					
220	200															01.60
					tat Tyr											2160
705	Der	1112	ASII	vai	710	116	mec	Ата	Asp	туs 715	GIN	гÀг	Asn	GIÀ	720	
. • •					, 10					113					720	
aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	ata	caq	2208
					Ile											
				725					730					735		
ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	ccc	gtg	2256
Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	Pro	Val	
			740					745					750			
					cac											2304
ьeu	ьeu		Asp	Asn	His	Tyr			Thr	Gln	Ser			Ser	Lys	
		755					760					765				
gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	gtg	acc	2352

57

Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr
770 775 780

gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag taa 2394
Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys *
785 790 795

<210> 16

<211> 797

<212> PRT

<213> Aequorea victoria and human

<400> 16

180

195

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185

Glu Leu Lys Ile Cys Arg Val Asn Arg Asn Ser Gly Ser Cys Leu Gly

200

190

Gly	Asp	Glu	Ile	Phe	Leu	Leu	Cys	Asp	Lys	Val	Gln	Lys	Glu	Asp	Ile
	210					215					220				
Glu	Val	Tyr	Phe	Thr	Gly	Pro	Gly	Trp	Glu	Ala	Arg	Gly	Ser	Phe	Ser
225					230					235					240
Gln	Ala	Asp	Val	His	Arg	Gln	Val	Ala	Ile	Val	Phe	Arg	Thr	Pro	Pro
				245					250					255	
Tyr	Ala	Asp	Pro	Ser	Leu	Gln	Ala	Pro	Val	Arg	Val	Ser	Met	Gln	Leu
			260					265					270		
Arg	Arg	Pro	Ser	Asp	Arg	Glu	Leu	Ser	Glu	Pro	Met	Glu	Phe	Gln	Tyr
		275					280					285			
Leu	Pro	Asp	Thr	Asp	Asp	Arg	His	Arg	Ile	Glu	Glu	Lys	Arg	Lys	Arg
	290					295					300				
Thr	Tyr	Glu	Thr	Phe	Lys	Ser	Ile	Met	Lys	Lys	Ser	Pro	Phe	Ser	Gly
305					310					315					320
Pro	Thr	Asp	Pro	Arg	Pro	Pro	Pro	Arg	Arg	Ile	Ala	Val	Pro	Ser	Arg
				325					330					335	
Ser	Ser	Ala	Ser	Val	Pro	Lys	Pro	Ala	Pro	Gln	Pro	Tyr	Pro	Phe	Thr
			340					345					350		
Ser	Ser	Leu	Ser	Thr	Ile	Asn	Tyr	Asp	Glu	Phe	Pro	Thr	Met	Val	Phe
		355					360					365			
Pro		Gly	Gln	Ile	Ser		Ala	Ser	Ala	Leu	Ala	Pro	Ala	Pro	Pro
	370	_				375					380				
	Val	Leu	Pro	Gln		Pro	Ala	Pro	Ala		Ala	Pro	Ala	Met	
385		_			390					395					400
Ser	Ala	Leu	Ala			Pro	Ala	Pro			Val	Leu	Ala		
_	_			405			_		410					415	
Pro	Pro	GIn		Val	Ala	Pro	Pro			Lys	Pro	Thr			Gly
<i>6</i> 3	<i>a</i> ,	mì	420		~ .		_	425		_			430		
GIU	стА		ьeu	Ser	GLu	Ala	Leu		GIn	. Leu	Gln		_	Asp	GLu
7	T	435	~ ~		-	~ 3	440		1	_	_	445			
Asp			Ala	Leu	Leu		Asn	Ser	Thr	Asp			Val	Phe	Thr
7 ~~	450		0	37 - 3	70	455		63	T)	0 3.	460		-	_	
		Ala	ser	vaı			Ser	GIU	Pne			ьеч	Leu	Asn	
465		D		7.7	470			m.		475		_			480
GTÀ	TTE	PIO	vaı			HIS	Thr	Thr			Met	. Leu	Met		
Dwo	C3	7.7	T1 -	485		.	**- 3	mı.	490			_	_	495	
FIO	GIU	Ата			Arg	ьeu	Val			Ala	GIN	Arg			Asp
D~^	71.7	D	500		. T	. C1	. 71 -	505		. .	. D	. 7) -	510		
FIO	чта			rro	ьес	ч стў	Ala		, GTZ	, ren	Pro			, ren	ı Let
		515					520					525	}		

Ser	Gly	Asp	Glu	Asp	Phe	Ser	Ser	Ile	Ala	Asp	Met	Asp	Phe	Ser	Ala
	530					535					540				
Leu	Leu	Ser	Gln	Ile	Ser	Ser	Leu	Asp	Pro	Pro	Val	Ala	Thr	Met	Val
545					550					555					560
Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	Val	Glu
				565					570					575	
Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	Glu	Gly
			580					585					590		
Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	Cys	Thr
		595					600					605			
Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	Leu	Thr
	610					615					620				
Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	Gln	His
625					630					635					640
Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	Arg	Thr
				645					650					655	
Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	Val	Lys
			660					665					670		
Phe	Glu		Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile	Asp
		675					680					685			
Phe		Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn	Tyr
	690					695					700				
	Ser	His	Asn	Val		Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	Gly	Ile
705		_			710					715					720
Lys	Vai	Asn	Phe		Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser		Gln
-		_		725					730					735	
ren	Ala	Asp		Tyr	GIn	Gln	Asn		Pro	Ile	Gly	Asp	-	Pro	Val
-		_	740	_				745					750		
Leu	Leu		Asp	Asn	His	Tyr		Ser	Thr	Gln	Ser		Leu	Ser	Lys
	_	755		_	_	_	760					765			
Asp		Asn	Glu	Lys	Arg		His	Met	Val	Leu		Glu	Phe	Val	Thr
7.1 -	770	C 3	T 3	m,		775		-		_	780	_	-		
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785					790					795					

<210> 17

<211> 2757

<212> DNA

<213> Aequorea victoria and human

130

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135

											gac					480
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155					160	
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
				165					170					175		
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180					185					190		_	
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctq	624
											Thr					
		195					200					205				
agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672
											Val	_	-	-		
	210					215					220					
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag	tcc	720
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Ser	
225					230					235					240	
gga	ctc	aga	tct	cga	ggc	aag	atg	gct	gac	ccg	gct	gcg	ggg	ccg	ccg	768
Gly	Leu	Arg	Ser	Arg	Gly	Lys	Met	Ala	Asp	Pro	Ala	Ala	Gly	Pro	Pro	
				245					250					255		
ccg	agc	gag	ggc	gag	gag	agc	acc	gtg	cgc	ttc	gcc	cgc	aaa	ggc	gcc	816
											Ala					
			260					265				_	270	_		
ctc	cgg	cag	aag	aac	gtg	cat	gag	gtc	aag	aac	cac	aaa	ttc	acc	gcc	864
											His					
		275					280		_			285				
cgc	ttc	ttc	aag	cag	ccc	acc	ttc	tgc	agc	cac	tgc	acc	gac	ttc	atc	912
											Cys					
	290		-			295		•			300				- 	

				cag Gln						960
				gtc Val				-	_	1008
				ccc Pro 345			_		_	1056
				ttt Phe					_	1104
				atg Met						1152
				aat Asn						1200
				atc Ile						1248
				aga Arg 425						1296
				ccc Pro						1344
				cag Gln						1392

tcc	ctc	aac	cct	gag	tgg	aat	gag	aca	ttt	aga	ttt	cag	ctg	aaa	gaa	1440
Ser	Leu	Asn	Pro	Glu	Trp	Asn	Glu	Thr	Phe	Arg	Phe	Gln	Leu	Lys	Glu	
465					470					475					480	
tcg	gac	aaa	gac	aga	aga	ctg	tca	gta	gag	att	tgg	gat	tgg	gat	ttq	1488
	Asp															
				485					490		•	•	•	495		
acc	agc	agg	aat	gac	ttc	atg	gga	tct	ttg	tcc	ttt	ggg	att	tct	gaa	1536
Thr	Ser	Arg	Asn	Asp	Phe	Met	Gly	Ser	Leu	Ser	Phe	Gly	Ile	Ser	Glu	
			500					505					510			
ctt	cag	aag	gcc	agt	gtt	gat	ggc	tgg	ttt	aag	tta	ctg	agc	cag	gag	1584
Leu	Gln	Lys	Ala	Ser	Val	Asp	Gly	Trp	Phe	Lys	Leu	Leu	Ser	Gln	Glu	
		515					520					525				
	ggc														-	1632
Glu	Gly	Glu	Tyr	Phe	Asn	Val	Pro	Val	Pro	Pro	Glu	Gly	Ser	Glu	Ala	
	530					535					540					
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	aat															1776
Asn	Asn	GLY		Arg	Asp	Arg	Met	Lys	Leu	Thr	Asp	Phe	Asn	Phe	Leu	
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	gtg															1824
Met	Val		Gly	Lys	Gly	Ser	Phe	Gly	Lys	Val	Met	Leu	Ser	Glu	Arg	
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Lys	Gly	Thr	Asp	Glu	Leu	Tyr	Ala	Val	Lys	Ile	Leu	Lys	Lys	Asp	Val	
	610					615					620					

		gac Asp 630					_			1920
		aag Lys							-	1968
		cgc Arg								2016
		cac His								2064
		gct Ala								2112
		att Ile 710				-			-	2160
		cac His					-	-	_	2208
		ggg Gly								2256
		gag Glu								2304
		ttt Phe								2352

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cag	gca	ccc	ttt	gaa	ggg	gag	gat	gaa	gat	gaa	ctc	ttc	caa	tcc	atc	2400
Gln	Ala	Pro	Phe	Glu	Gly	Glu	Asp	Glu	Asp	Glu	Leu	Phe	Gln	Ser	Ile	
785					790					795					800	
atg	gaa	cac	aac	gta	gcc	tat	ccc	aag	tct	atg	tcc	aag	gaa	gct	gtg	2448
Met	Glu	His	Asn	Val	Ala	Tyr	Pro	Lys	Ser	Met	Ser	Lys	Glu	Ala	Val	
				805					810					815		
gcc	atc	tgc	aaa	ggg	ctg	atg	acc	aaa	cac	cca	ggc	aaa	cgt	ctg	ggt	2496
Ala	Ile	Cys	Lys	Gly	Leu	Met	Thr	Lys	His	Pro	Gly	Lys	Arg	Leu	Gly	
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tgt	gga	cct	gaa	ggc	gaa	cgt	gat	atc	aaa	gag	cat	gca	ttt	ttc	cgg	2544
Cys	Gly	Pro	Glu	Gly	Glu	Arg	Asp	Ile	Lys	Glu	His	Ala	Phe	Phe	Arg	
		835					840					845				
•																
tat	att	gat	tgg	gag	aaa	ctt	gaa	cgc	aaa	gag	atc	cag	ccc	cct	tat	2592
Tyr	Ile	Asp	Trp	Glu	Lys	Leu	Glu	Arg	Lys	Glu	Ile	Gln	Pro	Pro	Tyr	
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										tcc			_			2640
	Pro	Lys	Ala	Arg	Asp	Lys	Arg	Asp	Thr	Ser	Asn	Phe	Asp	Lys	Glu	
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										act						2688
Phe	Thr	Arg	Gln		Val	Glu	Leu	Thr	Pro	Thr	Asp	Lys	Leu	Phe	Ile	
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										ttc						2736
Met	Asn	Leu		Gln	Asn	Glu	Phe	Ala	Gly	Phe	Ser	Tyr	Thr	Asn	Pro	
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Glu	Gly	Glu 35	Gly	Asp	Ala	Thr	Tyr 40	Gly	Lys	Leu	Thr	Leu 45	Lys	Phe	Ile
Cys	Thr 50	Thr	Gly	Lys	Leu	Pro 55	Val	Pro	Trp	Pro	Thr 60	Leu	Val	Thr	Thi
Leu 65	Thr	Tyr	Gly	Val	Gln 70	Cys	Phe	Ser	Arg	Tyr 75	Pro	Asp	His	Met	Lys
Gln	His	Asp	Phe	Phe 85	Lys	Ser	Ala	Met	Pro 90	Glu	Gly	Tyr	Val	Gln 95	Glu
Arg	Thr	Ile	Phe 100	Phe	Lys	Asp	Asp	Gly 105	Asn	Tyr	Lys	Thr	Arg 110	Ala	Glu
Val	Lys	Phe 115	Glu	Gly	Asp	Thr	Leu 120	Val	Asn	Arg	Ile	Glu 125	Leu	Lys	Gly
Ile	Asp 130	Phe	Lys	Glu	Asp	Gly 135	Asn	Ile	Leu	Gly	His 140	Lys	Leu	Glu	Туг
Asn 145	Tyr	Asn	Ser	His	Asn 150	Val	Tyr	Ile	Met	Ala 155	Asp	Lys	Gln	Lys	Asr
Gly	Ile	Lys	Val	Asn 165	Phe	Lys	Ile	Arg	His 170	Asn	Ile	Glu	Asp	Gly 175	Sei
Val	Gln	Leu	Ala 180	Asp	His	Tyr	Gln	Gln 185	Asn	Thr	Pro	Ile	Gly 190	Asp	Gly
Pro	Val	Leu 195	Leu	Pro	Asp	Asn	His 200	Tyr	Leu	Ser	Thr	Gln 205	Ser	Ala	Let
Ser	Lys 210	Asp	Pro	Asn	Glu	Lys 215	Arg	Asp	His	Met	Val 220	Leu	Leu -	Glu	Phe
Val 225	Thr	Ala	Ala	Gly	11e 230	Thr	Leu	Gly	Met	Asp 235	Glu	Leu	Tyr	Lys	Ser 240
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Leu	Arg	Gln 275	Lys	Asn	Val	His	Glu 280	Val	Lys	Asn	His	Lys 285	Phe	Thr	Ala

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Trp	Gly	Phe	Gly	Lys	Gln	Gly	Phe	Gln	Cys	Gln	Йаl	Суѕ	Cys	Phe	Val
305					310					315					320
Val	His	Lys	Arg	Cys	His	Glu	Phe	Val	Thr	Phe	Ser	Cys	Pro	Gly	Ala
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Asn	Val	His	Lys	Arg	Cys	Val	Met	Asn	Val	Pro	Ser	Leu	Cys	Gly	Thr
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Asp	His	Thr	Glu	Arg	Arg	Gly	Arg	Ile	Tyr	Ile	Gln	Ala	His	Ile	Asp
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Arg	Asp	Val		Ile	Val	Leu	Val	Arg	Asp	Ala	Lys	Asn	Leu	Val	Pro
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Met	Asp		Asn	Gly	Leu	Ser	Asp	Pro	Tyr	Val	Lys	Leu	Lys	Leu	Ile
_	_	435	_				440					445			
Pro		Pro	Lys	Ser	Glu		Lys	Gln	Lys	Thr	Lys	Thr	Ile	Lys	Суѕ
C	450	77 -	_	~ 3	_	455					460				
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465	7)	T	7.	_	470	_	_			475					480
ser	Asp	ьys	Asp		Arg	Leu	Ser	Val		Ile	Trp	Asp	Trp		Leu
Thr	Sor	7. ~~	7. ~	485	D1		0 3	_	490	_				495	
1111	Ser	Arg	500	Asp	Phe	Met	GTÀ		Leu	Ser	Phe	GLY		Ser	Glu
T.e.is	Gln	T.ve		Sar	17-1	7.00	C1	505	DL -	T	-		510		
Dea	CIII	515	ALG	Ser	val	Asp	520	rrp	Pne	гÀг	Leu		Ser	Gln	Glu
Glu	Glv		Tur	Phe	Δen	Val		Wal	Dro	Dma	<i>C</i> 3	525	0	G3	Ala
	530	Olu	ryr	1116	ASII	535	FIO	val	PIO	Pro		GTÀ	Ser	GIU	Ala
Asn		Glu	T.e.n	Ara	Gln		Pho	Clu	71 ~~~	21.0	540	T 7 -	0	01	Gly
545		O.L.u	LCu	111.9	550	БУЗ	rne	Giu	Arg		гÀг	ile	Ser	GIN	
	Lvs	Val	Pro	Glu	Glu	T.ve	Thr	Thr	Λen	555	17-1	C	T	Db -	560
			0	565	o Lu	Lys	* 117	+111	570	# 111T	vdl	ser	nλ2		ASP
Asn	Asn	G] v	Asn		Asn	Ara	Met	T.ve		ም ኤ ~	7	Dh-	7. ~	575 ph.	Leu
		- - 1	580	9	11010	****	430 L	585	neu	TILL	нзр	rne		rne	ьeu
Met	Val	Leu		Lvs	Gly	Ser	Phe		Lve	V=1	Mo+	Leur	590	G1	7\~~
		595		2 -			600	1	-,5	• 41		605	DET	GIU	ary

Lys	Gly 610	Thr	Asp	Glu	Leu	Tyr 615	Ala	Val	Lys	Ile	Leu 620	Lys	Lys	Asp	Val
Val 625	Ile	Gln	Asp	Asp	Asp 630	Val	Glu	Cys	Thr	Met 635	Val	Glu	Lys	Arg	Val 640
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Phe	Gln	Thr	Met 660	Asp	Arg	Leu	Tyr	Phe	Val	Met	Glu	Tyr	Val 670	Asn	Gly
Gly	Asp	Leu 675	Met	Tyr	His	Ile	Gln 680	Gln	Val	Gly	Arg	Phe	Lys	Glu	Pro
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Gln 705	Ser	Lys	Gly	Ile	Ile 710	Tyr	Arg	Asp	Leu	Lys 715	Leu	Asp	Asn	Val	Met 720
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Lys 865	Pro	Lys	Ala	Arg	Asp 870		Arg	Asp	Thr	Ser 875		Phe	e Asp	Lys	61u
Phe	Thr	Arg	Gln	Pro		Glu	Leu	Thr	Prc 890		Asp	Lys	: Leu	Phe	
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